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THE BIOLOGICAL LABORA

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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

Editorial Staff

E. G. Conklin—*Princeton University.*

Jacques Loeb—*The Rockefeller Institute for Medical Research.*

George T. Moore—*The Missouri Botanic Garden.*

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W. M. Wheeler—*Harvard University.*

E. B. Wilson—*Columbia University.*

Managing Editor

Frank R. Lillie—*The University of Chicago.*

Volume XXXIII.

WOODS HOLE, MASS.

JULY TO DECEMBER, 1917

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PRESS OF
THE NEW ERA PRINTING COMPANY
LANCASTER, PA.

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BIOLOGICAL BULLETIN

THE MARINE BIOLOGICAL LABORATORY

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I. TRUSTEES

AUGUST, 1916

EX OFFICIO

FRANK R. LILLIE, *Director*, The University of Chicago.
GILMAN A. DREW, *Assistant Director*, Marine Biological Laboratory.
D. BLAKELY HOAR, *Treasurer*, 161 Devonshire St., Boston, Mass.
GARY N. CALKINS, *Clerk of the Corporation*, Columbia University.

TO SERVE UNTIL 1920

H. H. DONALDSON ..... Wistar Institute of Anatomy and Biology.
M. J. GREENMAN ..... Wistar Institute of Anatomy and Biology.
C. W. HARGITT ..... Syracuse University.
H. S. JENNINGS ..... Johns Hopkins University.
GEORGE LEFEVRE ..... University of Missouri, *Secretary of the Board.*

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A. P. MATHEWS.....The University of Chicago.
G. H. PARKER.....Harvard University.
HENRY B. WARD.....University of Illinois.

TO SERVE UNTIL 1919

H. C. BUMPUS.....Tufts College.
R. A. HARPER.....Columbia University.
W. A. LOCY.....Northwestern University.
JACQUES LOEB.....The Rockefeller Institute for Medical Research.
F. P. MALL.....Johns Hopkins University.
GEORGE T. MOORE.....Missouri Botanical Garden, St. Louis.
L. L. NUNN.....Telluride, Colo.
JOHN C. PHILLIPS.....299 Berkeley Street, Boston, Mass.

TO SERVE UNTIL 1918

CORNELIA M. CLAPP.....Mount Holyoke College.
E. G. CONKLIN.....Princeton University.
ROSS G. HARRISON.....Yale University.
CAMILLUS G. KIDDER.....27 William Street, New York City.
M. M. METCALF.....Oberlin, Ohio.
WILLIAM PATTEN.....Dartmouth College.
JACOB REIGHARD.....University of Michigan.

TO SERVE UNTIL 1917

S. F. CLARKE.....Williamstown, Mass.
CHARLES A. COOLIDGE....Ames Building, Boston, Mass.
C. R. CRANE.....Woods Hole, Mass., *President of the Board.*
ALFRED G. MAYER.....Carnegie Institution.
C. E. MCCLUNG.....University of Pennsylvania.
T. H. MORGAN.....Columbia University.
ERWIN F. SMITH.....United States Department of Agriculture.
E. B. WILSON.....Columbia University.

II. ACT OF INCORPORATION

No. 3170.

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips

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and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory o· station fo· scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our LORD ONE THOUSAND, EIGHT HUNDRED AND EIGHTY-EIGHT.

HENRY B. PIERCE,
(Seal). *Secretary of the Commonwealth.*

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio*, members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The Director and Assistant Director, who shall be chosen by the Trustees, shall also be Trustees, *ex officio*.

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II. Special meetings of the members may be called by the Trustees, to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President and may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members of the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The President shall annually appoint two Trustees, who shall constitute a committee on finance, to examine from time to time the books and accounts of the Treasurer, and to audit his accounts at the close of the year. No investments of the funds of the Corporation shall be made by the Treasurer except approved by the finance committee in writing.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be given to the Boston Society of Natural History, or some similar public institution on such terms as may then be agreed upon.

IX. These By-Laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-Laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

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IV. THE TREASURER'S REPORT

STATEMENT OF AUDIT

February 28, 1917.

MR. D. BLAKELY HOAR,
161 Devonshire Street,
Boston.

Dear Sir: We have completed our audit of the accounts of the Marine Biological Laboratory for the year ended December 31, 1916, as kept both at your office in Boston and at Woods Hole and report thereon in the accompanying exhibits and schedules:

Exhibit *A*—Balance-Sheet as of December 31, 1916.
B—Income-and-Expense for Year ended December 31, 1916.

Schedule I—Investments (Book Values).
II—Cash Receipts and Payments on Account of Investments.
III—Summary of Inventory of Land, Buildings, and Equipment.
III*a*—Inventory of Land, Buildings, and Equipment, January 1, 1916.
IV—Balancing Account.

We certify that subject to the comments herewith the balance. sheet and income-and-expense statement shown herewith in Exhibits *A* and *B* are in accordance with the books and correct to the best of our knowledge and belief.

Very respectfully,
HARVEY S. CHASE & COMPANY,
Certified Public Accountants.

Note: Schedules II–IV are omitted in the following reports.

MARINE BIOLOGICAL LABORATORY BALANCE-SHEET, DECEMBER 31, 1916

Assets

Cash in Bank	$ 3,322.96	
Petty Cash at Woods Hole	200.00	$ 3,522.96
Accounts receivable		5,942.01
Investments (Schedule I)	$ 11,086.01	
Investment cash (Schedule II)	678.54	11,764.55

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Inventories (not available)		
Plant account (Schedule III):		
Land	$ 78,406.29	
Buildings	163,848.88	
Equipment	73,303.36	
	$315,558.53	
Less reserve for depreciation	6,942.12	308,616.41
		$329,845.93

Liabilities and Surplus

Accounts payable		$ 90.58
Suspense items		3.26
Notes payable	$ 3,000.00	
Turst funds	8,764.55	11,764.55
Mortgage on Gansett property		16,409.82
Balancing account		301,577.72
		$329,845.93

Marine Biological Laboratory Income and Expense for Year ended December 31, 1916

	Expense	Income	Loss	Gain
Administration expense	$ 6,923.92		$ 6,923.92	
Biological Bulletin	2,679.84	$ 1,534.08	1,145.76*	
Boat department expense	5,023.09		5,023.09	
Carpenter department	781.98	82.02	699.96	
Chemical department expense	1,455.45		1,455.45	
Dormitories	1,667.98	1,550.80	117.18	
Fish trap	1,348.68	1,406.92		$ 58.24
Interest on notes payable	150.00		150.00	
Instruction	4,075.70	5,075.00		999.30
Lectures, philosophical	100.00		100.00	
Lectures, evening	52.80		52.80	
Library department	3,447.37		3,447.37	
Mess	14,737.68	14,636.16	101.52	
Membership dues		980.00		980.00
Maintenance, buildings and grounds	6,180.18		6,180.18	
New Laboratory expense	2,636.11		2,636.11	
Pumping station expense	542.01		542.01	
Research income		3,650.00		3,650.00
Sundry expense and income	1,239.12	2,765.24		1,526.12
Supply department	13,838.43	21,096.65		7,258.22
Special dormitory expense, Dexter House	1,122.31		1,122.31	
Total expense	$68.002.65	$52,776.87	$29,697.66	$14,471.88
Total income	52,776.87		14,471.88	
Excess of expense	$15,225.78		$15,225.78	
Contribution by Mr. C. R. Crane	20,000.00			
Excess carried to balancing account	$ 4,774.22			

* Membership dues which are credited to the Biological Bulletin reduce the actual deficit to $165.76.

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Marine Biological Laboratory Investments (Book Values), December 31, 1916

Reserve Fund

$3,000 American Telephone & Telegraph Company, 4's (represented by receipts for same as collateral)	$2,921.25	
3/4 of $500 Western Telephone & Telegraph Company's 5's	372.66	
6 shares American Smelting & Refining Company Preferred (represented by receipts for same as collateral)	732.00	
8 shares General Electric Company	972.05	
14 shares United Shoe Machinery Company Preferred	393.75	
5 shares Massachusetts Gas Companies, Preferred	444.63	$ 5,836.34

Lucretia Crocker Fund

1/5 of $1,000 American Telephone & Telegraph Company, 4's	$ 194.75	
18 shares Vermont & Massachusetts Railroad Company	2,416.50	
1 share American Telephone & Telegraph Company	127.06	
1 share West End Street Railway Company	83.00	
2½ shares General Electric Company	323.85	3,145.16

Library Fund

4/5 of $1,000 American Telephone & Telegraph Company, 4's	$ 779.00	
1/4 of $500 Western Telephone & Telegraph Company, 5's	124.22	
3 shares American Telephone & Telegraph Company	381.18	
1 share American Smelting & Refining Company, Preferred	122.00	
2½ shares General Electric Company	288.10	
5 shares United Shoe Machinery Company, Preferred	140.63	
3 shares Massachusetts Gas Companies, Preferred	269.38	2,104.51
Total (Exhibit *A*)		$11,086.01

V. THE LIBRARIAN'S REPORT

August 8, 1916

As compared with last year, all numbers cataloged in the library make a total of 12,615. Of this total the following are new accessions:

Journals and serials from societies, laboratories, etc.	583
Books	84
Reprints	777
Last installment of A. M. N. H. loan	1,125

With the help of Prof. R. W. Tower, librarian of the American Museum of Natural History, who has been exceptionally generous in his coöperation, practically the entire loan has been placed on

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our shelves. Professor Tower has agreed to send to the library as received the current parts of 32 exchanges listed as follows:

Amer. Phil. Soc. Proc.,
Amsterdam, Verh. K. Akad. Wetenschappen,
Arkiv f. Botanik,
Arkiv f. Zoologi,
Ann. Soc. Zoöl. et Malacol. de Belgique,
Bull. Soc. Roy. de Botan. de Belgique,
Canadian Entomologist,
Bull. Int. Acad. Cracovie,
Dublin, Scientific Proc. Roy. Soc.,
Proc. Roy. Soc. Edinburgh,
Trans. and Proc. Botan. Soc. Edinburgh,
Berichte d. Naturf. Gesell. Freiburg,
Journ. Linnean Soc. London,
Proc. Roy. Soc. London, Ser. B,
Trans. Zoöl. Soc. London,
Mem. and Proc. Manchester Literary and Phil. Soc.,
Trans. and Rep. Manchester Micr. Soc.,
Annales du Mus. d'Hist. Nat. Marseilles,
Medd. K. Vetenskapsakad. Nobelinstitut,
Journ. N. Y. Botan. Gard.,
Bull. Mus. Hist. Nat. Paris,
Atti d. Soc. Toscana Pisa,
Sitzungsber. d. K. B. ges. d. wiss. Math. Natur. Classe, Prag.,
Bull. Acad. Imp. Sci. St. Petersburg,
Memoirs Acad. Imp. Sci. St. Petersburg,
Stettiner Entomol. Zeitung,
K. Svenska Ver. Akad. Aarsbok,
K. Svenska Vet. Akad. Handlingar,
Boll. Mus. Zoöl. Comp. Anat. Torino,
Sitzungsber. Akad. wiss. Math. Naturw. Klasse, Wien,
Verhandlungen K. K. Zool. Bot. Gesell., Wien.

2,269 volumes and parts of volumes have been accessioned. 867 of these were bound when received. In order to care for the remainder, 656 volumes had to be bound this year, which about doubled our usual binding bill, adding to it $667.59. (This with

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$171.90 previously expended for a former installment of this loan, makes a total cost for binding of $839.49.) In addition to the above, the American Museum has also given about 1,000 pamphlets that have not been cataloged.

A considerable addition will be made to the library through the sale of duplicate material representing a value of about $350. The details of this will be announced later.

A number of gifts have been made to the library which are greatly valued. From publishers, whose works are especially well placed for examination here, we have received the following:

Open Court Publishing Co., 2 books,
Cambridge University Press, 31 books,
Saunders, 1 book,
University of Chicago Press, 2 books.

Books have been received from authors as follows: A. P. Mathews, C. M. Child, J. M. Coulter, C. F. Chamberlain, M. F. Guyer, F. D. Woods, H. H. Donaldson, Henry Kraemer, C. J. Herrick, J. F. Abbott, H. E. Jordan, J. S. Ferguson, and Ramon y Cajal.

Miss Foot presented a long series of papers by Boveri and Whitman and Dr. F. R. Lillie has given 10 miscellaneous volumes.

777 reprints have been given by a long list of authors, among whom are especially to be mentioned 19 English writers: Wm. Bateson, L. Doncaster, E. S. Goodrich, W. Woodland, A. D. Cotton, W. M. Tattersall, W. E. Shipley, R. Gurney, T. V. Hoggson, Sir Charles Elliot, R. C. Punnett, T. Johnson, Richard Elmhist, J. F. Gemmill, F. Jeffrey Bell, W. B. Benham, A. Willey, W. A. Herdman, and Arnold T. Watson.

Occasion is taken here to thank the other numerous donors whose names may not be given for lack of space.

Among miscellaneous gifts are 16 volumes of the *Biochemische Zeitschrift* from Dr. A. R. Moore, which will be of great value in starting this new set. The Wistar Institute Monographs have been presented by Dr. Greeman, and the Rockefeller Institute has given a set of the Rockefeller Institute Studies. The Surgeon-General's Library has also presented Series 2 of the Index Catalog, which fills a long felt need.

Dr. Drew has deposited with the library his valuable collection of reprints and these have been in constant use this summer.

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Under the head of journals and serials from museums, academies, laboratories, etc., the following sets have been completed this year:

Journal of Medical Research,
Zeit. f. Biochem. u. Biophysik,
Kansas University Science Bulletin.

223 volumes have been added during the year to incomplete sets.

The 31 serials listed below were not on our shelves last year:

Univ. Calif. Pub. in Physiology,
Univ. Cal. Pub. in Botany,
Bull. Soc. Roy. de botan. de Belgique,
Mem. Soc. Roy. de Liége,
Bull. Mus. d'hist. nat. Paris,
Abh. Naturwiss. Vereins zu Bremen,
Mem. and Proc. Manchester Literary and Phil. Soc.,
Proc. Roy. Soc. London, Sec. B,
Verhandlungen Zool. Botan. Gesell. Wien,
Sitzungsber. K. Akad. wiss. Wien,
Mem. Soc. Nat. Kiew,
Sitzungsber. Naturforsch. Gesell. Dorpat,
Ann. Mus. Zool. Acad. Imp. Sci. St. Petersburg,
Bull. Acad. Imp. Sci. St. Petersburg,
Mem. Acad. Imp. Sci. St. Petersburg,
Annals Scottish Nat. Hist. Soc.,
Proc. Roy. Soc. Edinburgh,
Sitzungsber. K. B. Gesell. wiss. Prag,
Verh. Natur. wiss. Vereins Heidelberg,
Math. u. Naturwiss. Berichte aus Ungarn,
Berichte d. Naturfor. Gesell. Freiburg,
Verh. K. Akad. Wetensch. Amsterdam,
Proc. Roy. Dublin Soc.,
Atti Soc. Toscana Pisa,
Boll. Mus. Zool. Comp. Anat. Torino,
K. Svenska Vet. Akad. Aarsbok,
K. Svenska Vet. Akad. Handlingar,
Medd. Vet. Akad. Noberinstitut.

We have also entered subscriptions for new journals as follows:

Zeit. f. induk. abstamm. u. vererbungslehre,

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Genetics,
Journ. Med. Res.,
Physiological Abstracts,
Biochem. Zeitschrift.

One new exchange with the BIOLOGICAL BULLETIN has been arranged: *Bulletin de la Societé Zool. de France.*

Dr. H. H. Donaldson has generously sent a check to cover the cost of a new Century Dictionary, which has been greatly needed.

Attention is called here to our collection of photographs of foreign laboratories secured by exchange. It is planned to extend this collection to include other laboratories.

It was pointed out last year that four years ago a fund was started to aid us in extending the journal files. Last year the subscriptions to this fund had reached the sum of $91. This year a much larger group of investigators (60) whose names follow, continuing Dr. Tashiro's lead, contributed in amounts from 50 cents to $2.00. A total of $53.00 has been received in this way to date.

W. C. Allee,
Ezra Allen,
F. H. Adler,
G. A. Baitsell,
R. H. Bowen,
C. B. Bridges,
R. A. Budington,
G. L. Carver,
Eleanor Carothers,
C. M. Clapp,
C. M. Child,
C. L. Chapin,
E. J. Cohn,
M. Copeland,
E. V. Cowdry,
I. J. Davies,
G. A. Drew,
D. J. Edwards,
W. E. Garrey,
R. S. Lillie,
Jacques Loeb,
E. J. Lund,
Clara J. Lynch,
E. P. Lyon,
E. E. MacMorland,
C. E. McClung,
T. H. Morgan,
Stewart Mudd,
W. J. V. Osterhout,
Charles Packard,
B. M. Patten,
C. L. Parmenter,
Florence Peebles,
A. Richards,
Miss A. E. Richards,
C. G. Rogers,
F. M. Root,
A. F. Shull,

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Wilson P. Gee,	R. A. Spaeth,
A. J. Goldfarb,	A. H. Sturtevant,
H. B. Goodrich,	H. D. Taylor,
A. O. Gross,	Clara L. Ware,
M. F. Guyer,	M. W. Welch,
L. V. Heilbrunn,	D. H. Wenrich,
Mary J. Hogue,	Paul S. Welch,
E. R. Hoskins,	H. L. Wieman,
J. S. Huxley,	W. H. Weston,
F. R. Lillie,	Miss A. E. Woodward.

Dr. Bumpus contributed $25 and Doctors Meigs, A. G. Mayer, and Knower added $10 each toward completing the sums previously promised by them. In this way the total amount of $108 was received this year. In addition there are still a few subscriptions expected from promises already published.

This fund represents a most important coöperative spirit in the development of the library. The library is used more now than ever before, as witnessed by the fact that 350 books were charged in one day. Never before have so many individuals of the Laboratory shown their appreciation of the library by voluntarily contributing to its support and extension. Such wide interest on the part of so many members of the Laboratory leading to numerous small gifts, whether in money or books, will be of the greatest value to the library. It is much appreciated! All the more because it is so broadly coöperative!

It is to be hoped that the Laboratory can soon supplement these gifts and place at the disposal of the library a sum to be expended in emergencies for the purchase of bargains which often become unexpectedly available. Such a fund of say $300 a year would make it possible to fill in gaps, complete sets, and add works which would be difficult to secure through more regular and slower channels. There is no more urgent need of the library than the maintenance of such an emergency fund, now so well started.

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VI. THE DIRECTOR'S REPORT

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I have the honor to present a report of the twenty-ninth session of the Marine Biological Laboratory for the year 1916. The total attendance was slightly smaller than in 1915, 231 as compared with 242, but larger than that of any earlier year. There were 129 investigators and 102 students, a falling off of 8 in the number of investigators and of 3 in the number of students. The decrease in investigators falls entirely under the head of investigation under instruction; indeed the number of independent investigators shows a gain of 5. The decrease should therefore be regarded as probably of a fluctuating character, as the group concerned consists largely of research students of members of the independent group of investigators. The slight decrease in student attendance was due entirely to our method of registration, for the number of applicants for courses was larger than ever before; after the courses were registered to the limit of our accommodations many students were refused registration. Later some of those registered failed to appear. It is obvious, therefore, that heads of departments should be allowed to register in excess of accommodations to a certain extent, in the expectation that a certain proportion of those registered will finally be unable to attend. The past season is the first one in which registration in advance was positively required, and this was done because the experience of 1915 had shown that unlimited registration was likely to exceed capacity, as indeed the number of applications in 1916 actually showed.

In spite of the slight decrease in numbers there was an increase in the receipts from investigators and students over all previous sessions, viz.: $8,725 in 1916 as compared with $8,325 in 1915, $7,300 in 1914, $6,160 in 1913, and $5,175 in 1912. The gain was entirely from the research side due to the fact that coöperating institutions subscribed $800 more in 1916 than in 1915 ($5,000 as against $4,200). The number of subscribing institutions was 40 in 1916 and 37 in 1915. The Supply Department has again shown a large increase of sales, which amounted to $21,096.65,

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as against $16,932.00 in 1915, $14,003.35 in 1914, $14,554.40 in 1913, $13,966.35 in 1912, $10,303.61 in 1911. The business has actually doubled in the last six years.

For several years the Director has called attention to need of increased accommodation for workers at the Laboratory in two directions: first the need of extra dormitory space for students, and second the need of cottages for the families of investigators, or of land on which such cottages could be placed. Both these needs now bid fair to be satisfied for some years to come. Mr. Crane has turned over to the Laboratory the Dexter House for use as a dormitory, with a sufficient sum of money to repair and equip it. We feel that with the other dormitory accommodations previously provided the students' needs will be adequately cared for. At the same time this extra accommodation enables us to dedicate the third floor of the Supply Department building, hitherto reserved for dormitory purposes, to the rapidly growing needs of this Department.

Provision has also been made for building sites for investigators by the purchase of 21 acres of woodland in the Gansett woods, immediately joining the Crow Hill region of Woods Hole, in which many of the Laboratory members now have homes, and extending from Quissett Ave. nearly to Buzzards Bay. This piece of land enjoys perpetual rights in a bathing reservation in a sandy cove of Buzzards Bay immediately adjoining. For convenience of location with reference to the Laboratory and to the sea no more desirable location could have been found; and there is no more beautiful tract in the entire town of Falmouth. Mr. Crane has furnished the money necessary to complete the purchase on favorable terms made by the owners, and he has also provided funds for its development, including the building of roads, which it is hoped may be accepted by the town. This work is now in progress, so that it is expected that parts of the land will be available for building purposes in the spring. A considerable number of requests for lots already have been received and some preliminary assignments made.

In view of the difficulty that has existed for so long in securing building sites for our membership and the probability that such difficulties are likely to increase in the future, the director

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recommends that none of this land be permanently alienated from the Laboratory by outright sale; and in order that it may be reserved for the purposes for which it was acquired he also recommends that none of it be otherwise disposed of except to members of the Laboratory unless by vote of the Board of Trustees in each case. Deeds of sale should, therefore, include a claim ensuring ultimate reversion to the Laboratory on equitable terms, and provisions should also be made for long term leaseholds. The form and value of the leasehold have occasioned much thought, and certain proposals are herewith presented for consideration by the Board. The valuations have been determined with no idea of speculative gain, but solely from the point of view of covering cost and expense of development with a small margin of safety for probable slowness of development. There is room obviously for difference of opinion with reference to values set on individual lots which have been determined as nearly as possible on the basis of general desirability. It will be found on comparison that the values thus determined contrast very favorably with the prices of building lots anywhere else in Woods Hole.

There is appended as part of this report a list of the staff, of investigators and students in attendance, a tabular view of attendance covering the last five years, a list of subscribing institutions, the Evening Lectures, and the Membership of the Corporation.

I. THE STAFF

1916

FRANK R. LILLIE, DIRECTOR,
Professor of Embryology, and Chairman of the Department of Zoölogy, The University of Chicago.

GILMAN A. DREW, ASSISTANT DIRECTOR, Marine Biological Laboratory.

ZOÖLOGY

I. INVESTIGATION

GARY N. CALKINS.....Professor of Protozoölogy, Columbia University.

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E. G. CONKLIN.....Professor of Zoölogy, Princeton University.

GILMAN A. DREW.....Assistant Director, Marine Biological Laboratory.

GEORGE LEFEVRE.....Professor of Zoölogy, The University of Missouri.

FRANK R. LILLIE.....Professor of Embryology, The University of Chicago.

C. E. MCCLUNG.....Professor of Zoölogy, University of Pennsylvania.

T. H. MORGAN.....Professor of Experimental Zoölogy, Columbia University.

E. B. WILSON.....Professor of Zoölogy, Columbia University.

II. INSTRUCTION

CASWELL GRAVE.....Associate Professor of Zoölogy, Johns Hopkins University.

W. C. ALLEE.....Professor of Biology, Lake Forest College.

GEORGE A. BAITSELL.....Instructor in Biology, Yale University.

ROBERT HALL BOWEN....Graduate Student, Columbia University.

W. J. KOSTIR.....Instructor in Zoölogy and Entomology, Ohio State University.

T. W. PAINTER.....Adjunct Professor of Zoölogy, University of Texas.

FRANCIS M. ROOT.....Bruce Fellow, Johns Hopkins University.

EMBRYOLOGY

I. INVESTIGATION (See Zoölogy)

II. INSTRUCTION

WILLIAM E. KELLICOTT...Professor of Biology, Goucher College.

ROBERT A. BUDINGTON....Professor of Zoölogy, Oberlin College.

CHARLES PACKARD.....Instructor in Zoölogy, Columbia University.

CHARLES G. ROGERS.....Professor of Zoölogy, Oberlin College.

REYNOLD A. SPAETH.....Instructor in Biology, Yale University.

PHYSIOLOGY

I. INVESTIGATION

ALBERT P. MATHEWS.....Professor of Physiological Chemistry, The University of Chicago.

RALPH S. LILLIE.....Professor of Biology, Clark University.

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HAROLD C. BRADLEY.....Assistant Professor of Physiological Chemistry, University of Wisconsin.

SHIRO TASHIRO.....Instructor in Physiological Chemistry, The University of Chicago.

II. INSTRUCTION

RALPH S. LILLIE.....Professor of Biology, Clark University.

WALTER E. GARREY.....Professor of Physiology, Tulane University.

FRANK P. KNOWLTON.....Professor of Physiology, Syracuse University.

A. R. MOORE.....Professor of Physiology, Rutgers College.

PHILOSOPHICAL ASPECTS OF BIOLOGY AND ALLIED SCIENCES

LECTURES

EDWARD G. SPAULDING....Professor of Philosophy, Princeton University.

BOTANY

GEORGE T. MOORE.....Director, Missouri Botanical Garden and Professor of Botany, Washington University.

IVEY F. LEWIS.....Professor of Biology, University of Virginia.

CARL S. HOAR.....Instructor in charge of Botany, Williams College.

LIBRARY

H. E. MCKNOWER.....Professor of Anatomy, University of Cincinnati, Librarian.

MARY E. SCOTT.....Assistant Librarian.

Chemical Supplies[1]

OLIVER S. STRONG.....Instructor in Anatomy, College of Physicians and Surgeons, New York City, Chemist.

Supply Department

G. M. GRAY.....Curator.

JOHN J. VEEDER....Captain.

E. M. LEWIS.....Engineer.

A. W. LEATHERS...Collector.

A. M. HILTON.....Collector.

F. G. GUSTAFSON...Collector in Botany.

EDNA E. WELLS....Clerk.

[1] Compound microscopes, with two oculars and two objectives, rack and pinion and fine adjustment, may be rented for the seasons at $7.00 each, provided notice is received by the assistant director not later than June 15.

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F. M. MacNaught.....Business Manager.
Herbert A. Hilton....Superintendent of Buildings and Grounds.

2. INVESTIGATORS AND STUDENTS

1916

ZOÖLOGY

Independent Investigators

Addison, William H. F., Assistant Professor of Normal Histology and Embryology, University of Pennsylvania.
Allee, Warder C., Professor of Biology, Lake Forest College.
Allen, Ezra, Honorary Fellow, University of Pennsylvania.
Baitsell, George A., Instructor in Biology, Yale University.
Binford, Raymond, Professor of Zoölogy, Earlham College.
Bridges, Calvin B., Columbia University.
Budington, Robert A., Professor of Zoölogy, Oberlin College.
Calkins, Gary N., Professor of Protozoölogy, Columbia University.
Carothers, E. Eleanor, University of Pennsylvania.
Carver, Gail L., Professor of Biology, Mercer University.
Chidester, Floyd E., Associate Professor of Zoölogy, Rutgers College.
Child, Charles M., Associate Professor of Zoölogy, University of Chicago.
Clapp, Cornelia M., Professor Emeritus, Mount Holyoke College.
Conklin, E. G., Professor of Biology, Princeton University.
Copeland, Manton, Professor of Biology, Bowdoin College.
Cowdry, Edmund V., Associate in Anatomy, Johns Hopkins Medical School.
Cowdry, N. H., Johns Hopkins Medical School.
Crampton, H. E., Professor of Zoölogy, Barnard College, Columbia University.
Donaldson, Henry H., Professor of Neurology, Wistar Institute.
Drew, Gilman A., Assistant Director, Marine Biological Laboratory, Woods Hole.
Gee, Wilson, Professor of Biology, Emory University.
Goldfarb, A. J., Assistant Professor of Biology, College of the City of New York.
Goldschmidt, Richard, Kaiser Wilhelm Institut für Biologie.
Goodrich, Hubert B., Instructor in Biology, Union College.
Grave, Caswell, Associate Professor of Zoölogy, Johns Hopkins University.
Gregory, Emily R., University of Pennsylvania.
Gross, Alfred O., Assistant Professor of Zoölogy, Bowdoin College.
Guyer, Michael F., Professor of Zoölogy, University of Wisconsin.
Heilbrunn, Lewis V., Associate in Embryology, University of Chicago.
Hogue, Mary J., Instructor in Zoölogy, Wellesley College.
Hoskins, Elmer R., Instructor in Anatomy, New York University.
Jacobs, Merkel H., Assistant Professor of Zoölogy, University of Pennsylvania.
Kellicott, William E., Professor of Biology, Goucher College.
Kernall, Morris J., University of Illinois.
Knower, Henry McE., Professor of Anatomy, University of Cincinnati.
Lefevre, George, Professor of Zoölogy, University of Missouri.
Lillie, Frank R., Professor of Embryology, University of Chicago.
Lund, Elmer J., Assistant Professor of Zoölogy, University of Minnesota.

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McClung, Clarence E., Director of Zoölogy Laboratories, University of Pennsylvania.
Malone, Edward F., Associate Professor of Anatomy, University of Cincinnati.
Moodie, Roy L., Associate in Anatomy, University of Illinois.
Moore, Carl R., Assistant in Embryology, University of Chicago.
Morgan, Thomas H., Professor of Experimental Zoölogy, Columbia University.
Morris, Margaret, Yale University.
Muller, Hermann J., Instructor, Rice Institute.
Newman, Horatio H., Associate Professor of Zoölogy, University of Chicago.
Packard, Charles, Instructor in Zoölogy, Columbia University.
Pappenheimer, Alvin M., Assistant Professor of Pathology, Columbia University.
Patten, William, Professor of Biology, Dartmouth College.
Peebles, Florence, Professor of Biology, H. Sophie Newcomb Memorial College.
Reagen, F. P., Princeton University.
Richards, A., Adjunct Professor of Zoölogy, University of Texas.
Rogers, Charles G., Professor of Comparative Physiology, Oberlin College.
Shull, A. Franklin, Associate Professor of Zoölogy, University of Michigan.
Shumway, Waldo, Instructor in Biology, Amherst College.
Spaeth, Reynold A., Instructor in Biology, Yale University.
Spaulding, Edward G., Professor of Philosophy, Princeton University.
Stockard, Charles R., Professor of Anatomy, Cornell Medical College.
Strong, Oliver S., Instructor in Anatomy, Columbia University.
Strong, Archibald M., Instructor in Medicine, Columbia University.
Sturtevant, Alfred H., Columbia University.
Taylor, Herbert D., Assistant in Pathology and Bacteriology, Rockefeller Institute.
Tennent, David H., Professor of Biology, Bryn Mawr College.
Wenrich, David H., Instructor in Zoölogy, University of Pennsylvania.
Weinstein, Alexander, Fellow, Columbia University.
Welch, Paul S., Associate Professor of Entomology, Kansas State Agricultural College.
Whiting, Phineas W., Research Fellow, University of Pennsylvania.
Wieman, Harry L., Head of Dept. of Zoölogy, University of Cincinnati.
Wilson, E. B., Professor of Zoölogy, Columbia University.
Woodward, Alvalyn E., Instructor, Vassar College.

Beginning Investigators

Blake, Irving H., Dept. of Zoölogy and Physiology, Oregon Agricultural College.
Brumfiel, Daniel M., Instructor in Animal Biology, University of Iowa.
Chapin, Catharine L., University of Chicago.
Cohn, Edwin J., University of Chicago.
Davies, I. J., Micrologist, Rice Institute.
Globus, Joseph H., Assistant in Anatomy, Cornell Medical School.
Goldman, Agnes, Cornell Medical School.
Heilbrunn, Stella, Teacher, Bay Ridge High School, Brooklyn.
Herman, William, Harvard Medical School.
Hoy, William E., Jr., Instructor, University of Rochester.
Huxley, Julian S., Assistant Professor of Biology, Rice Institute.
Lynch, Clara J., 925 W. Cleveland Ave., Canton, Ohio.

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MacArthur, John W., Associate in Zoölogy, University of Chicago.
MacMorland, Edward E., Fellow in Biology, Princeton University.
Cooper, Arthur R., Fellow in Zoölogy, University of Illinois.
Minoura, Tadachika, University of Chicago.
Montgomery, Mrs. Priscilla B., University of Pennsylvania.
Parmenter, Charles L., Assistant, University of Minnesota.
Pinney, Mary E., Demonstrator in Biology, Bryn Mawr College.
Root, Francis M., Fellow, Johns Hopkins University.
Safir, Shelley R., Teacher in Stuyvesant High School, New York City.
Smith, Christianna, Assistant, Zoölogy Department, Mt. Holyoke College.
Speidel, Carl C., Graduate Student, Princeton University.
Ware, Clara C., Assistant at Barnard College, Columbia University.
White, Edith G., Research Assistant, Princeton University.

PHYSIOLOGY

Independent Investigators

Chambers, Robert, Jr., Instructor in Anatomy, Cornell Medical College.
Day, Edward C., Lecturer in Physiology, Bryn Mawr College.
Duesberg, Jules, Research Associate, Carnegie Institution.
Edwards, Dayton J., Instructor in Physiology, College of City of New York.
Ellis, Frederick W., Monson, Mass.
Garrey, Walter E., Professor of Physiology, Tulane University.
Harvey, E. Newton, Assistant Professor of Physiology, Princeton Univ.
Harvey, Mrs. E. Newton, Princeton, N. J.
Knowlton, Frank P., Professor of Physiology, Syracuse University.
Kudo, R., Rockefeller Institute for Medical Research.
Lillie, Ralph S., Professor of Biology, Clark University.
Loeb, Jacques, Head of Department of Experimental Biology, Rockefeller Institute for Medical Research.
Lyon, Elias P., Professor of Physiology, University of Minnesota.
Mathews, Albert P., Professor of Physiological Chemistry, University of Chicago.
Moore, Arthur R., Professor of Physiology, Rutgers College.
Noguchi, Hideyo, Research Member, Rockefeller Institute for Medical Research.
Northrop, John H., Assistant, Rockefeller Institute for Medical Research.
Patten, Bradley M., Senior Instructor, Western Reserve Medical School.
Price, Weston A., Chairman, Research Commission of the National Dental Association, Cleveland, Ohio.
Richards, Annabella E., Demonstrator of Chemistry, Woman's Medical College of Pennsylvania.
Rudisch, Julius, Consulting Physician, Mount Sinai Hospital, New York.
Van der Stricht, O., Western Reserve Medical School.
Wasteneys, Hardolph, Associate in Experimental Biology, Rockefeller Institute for Medical Research.

Beginning Investigators

Adler, Francis H., University of Pennsylvania.
Chamberlain, Mary M., Fellow, University of Pennsylvania.
Noble, Arlyle, Assistant in Bacteriological Research, Parke Davis & Co.

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BOTANY

Independent Investigators

LEWIS, IVEY F., Professor of Biology, University of Virginia.
LYMAN, GEORGE R., Pathological Inspector, Dept. of Agriculture, Washington, D. C.
MOORE, GEORGE T., Director, Missouri Botanical Garden, St. Louis, Mo.
MOORE, EMMELINE, Instructor, Vassar College.
OSTERHOUT, W. J. V., Professor of Botany, Harvard University.
SNOW, LAETITIA M., Associate Professor of Botany, Wellesley College.
WESTON, WILLIAM H., JR., Instructor in Biology, Adelbert College.

Beginning Investigator

HAAS, ALBERT R., Graduate Student, Harvard University.

STUDENTS

1916

BOTANY

BROWNING, HAROLD W., University of Wisconsin, Madison, Wis.
CLUM, HAROLD H., Student, Oberlin College, Oberlin, Ohio.
DONOVAN, STEPHEN M., Student, St. Bonaventure, St. Bonaventure, N. Y.
DE ANGULO, MRS. CARY F., Carmel, California.
FURBER, JANE M., 769 Washington St., Brookline, Mass.
JONES, WINIFRED, Teacher, Cincinnati Public Schools, Cincinnati, Ohio.
MCNAIR, FRANCES D., Smith College, Northampton, Mass.
MCWHORTER, FRANK P., Vanderbilt University, Nashville, Tenn.
MARTIN, GEORGE W., Instructor, Mass. Agricultural College, Amherst, Mass.
PAGE, FREDERICK S., Instructor, Dartmouth College, Hanover, N. H.
REEVES, WALTER S., Missouri Botanical Garden, St. Louis, Mo.
SMITH, EDNA L., Carmel, California.

EMBRYOLOGY

BAKER, ETHEL C., 123 Chiswick Road, Boston, Mass.
BEYER, HENRY G., U. S. Navy, Retired.
BODINE, JOSEPH H., Assistant in Zoölogy, University of Pennsylvania.
BOWEN, ROBERT H., 330 Summit Ave., Mt. Vernon, N. Y.
CHARLTON, HARRY H., North Billerica, Mass.
DRUIEN, MILDRED M., Vassar College, Poughkeepsie, N. Y.
HAGUE, FLORENCE, Assistant, Kansas State Agricultural College.
HOAGLAND, RUTH A., Assistant in Zoölogy, Vassar College.
HOPKINS, HOYT S., Johns Hopkins University, Baltimore, Md.
JOB, THESLE T., Fellow, State University of Iowa.
LOPEZ-SUAREZ, JUAN, Madrid.
MCROBERTS, EARL S., Butler College, Indianapolis, Ind.
MAHR, ERNEST F., Syracuse University, Syracuse, N. Y.
MASON, MICHAEL L., Northwestern University.
MOORE, DOROTHEA M., Radcliffe College, Cambridge, Mass.

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MYERS, DOROTHY K., Barnard College, New York City.
PARKER, SYLVIA L., Laboratory Assistant, Mt. Holyoke College.
RAE, ANNE M., Teacher, Girls High School, Brooklyn, N. Y.
RIPPLE, C. VINCENT, Instructor, South Philadelphia High School.
ROWLAND, EDITH L., Barnard College, New York City.
SCHNEIDER, PETER A., Instructor, University of Vermont.
SHAW, MYRON E., Student, Carleton College.
SMITH, ELIZABETH A., Instructor in Zoölogy, University of Wisconsin.
SPROWLES, EDITH A., Teacher, Frankford High School, Frankford, Pa.
VAUPEL, JEAN, University of Cincinnati.
WARDWELL, EDWARD H., Graduate Student, Brown University.

PHYSIOLOGY

CHASE, SAMUEL W., Student, Harvard University.
CHILDS, HENRY E., Teacher, Rochester, N. Y.
GREISHEIMER, ESTHER, Clark University.
HUNT, HARRISON R., Student, Harvard University.
IRWIN, MARION, 53 Garden St., Cambridge, Mass.
MILLIKIN, FRANCES, Smith College, Northampton, Mass.
MUDD, STUART, Princeton, N. J.
MÜHL, ANITA M., Butler College, Indianapolis, Ind.
POND, SAMUEL E., Fellow, Clark University.
RUNYON, PAUL M., Student, Princeton University.
SAMPSON, MYRA M., Instructor, Smith College.
TWINING, RALPH H., Clark University.
YOUNG, PAUL T., Graduate Student, Princeton University.
YOUNG, ELDRID G., 4876 Sherbrooke St., Montreal.

ZOÖLOGY

BACH, ELLEN B., 721 S. Ingalls St., Ann Arbor, Mich.
BARGEN, JACOB A., Student, Carleton College.
BICKERSTAFF, FLORENCE W., Student, Oberlin College.
BIDDLE, M. GEORGINA, 2017 De Lancey Place, Philadelphia, Pa.
BLATCHFORD, BARBARA, Student, Vassar College.
BRENTON, THADDEUS R., Laboratory Assistant in Zoölogy, Moores Hill College.
BRUMBACK, FLORENCE M., Teacher, Waukegan Township High School.
BRUNSWICK, MARK, Quissett, Mass.
BRYANT, GLADYS, Student, Radcliffe College.
CHOATE, MABEL, Barnard College, Columbia University.
CAULDWELL, OLIVIA, Student, Vassar College.
COLVILL, HELEN, Teacher, Circleville, Ohio.
DAVIS, GORDON E., Oberlin College.
DAWSON, JAMES A., Assistant in Biology, Yale University.
DETWILER, SAMUEL R., Graduate Student, Yale University.
DE LA CRUZ, Dionysia G., Student, Sophie Newcomb College.
ELLINGER, MARGARET, 14 W. 25th St., Baltimore, Md.
FELTY, AUGUSTUS R., 734 Prospect Ave., Hartford, Conn.
HERRICK, RUTH, University of Chicago.
HINRICHS, MARIE A., 1832 Blue Island Ave., Chicago, Ill.

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HOULDING, ERNEST W., 182 E. College St., Oberlin, Ohio.
LAMB, LAURA, 527 S. 41st St., Philadelphia, Pa.
LEVIEN, EUNICE M., Lake Forest College.
LEIGH, HOWARD, Earlham College,
MACK, RUTH J., Radcliffe College, Cambridge, Mass.
NEUN, ELSIE G., University of Rochester.
NUTE, BERTHA E., Mount Holyoke College.
ROACH, ETHEL S., University of Rochester.
RORER, MARY D., Student, Mount Holyoke College.
ROWE, GEORGIA M., 2321 N. Calvert St., Baltimore, Md.
RUHL, REBECCA L., 205 E. Main St., Clarksburg, W. Va.
SEAGO, MARY M., Student, Sophie Newcomb College.
SKILLERN, INEZ, Sweet Briar College, Sweet Briar, Va.
STEWART, ELEANOR G., 425 St. Clair St., Pittsburgh, Pa.
STEWART, FRED W., Schuyler Fellow, Cornell University.
SWANN, MRS. ARTHUR W., 124 East 61st St., New York City.
TAYLOR, MARTHA, Student, Radcliffe College.
TILGHMAN, SARAH A., Goucher College.
TURK, IRENE, Hunter College, New York City.
VERNOOY, LESTER C., Amherst College.
WAKEFIELD, LUCILE, Student, Carnegie Institute of Technology.
WALKER, LOUISE, Laboratory Assistant, Brearley School, New York City.
WALTERS, MARY J., Goucher College, Baltimore, Md.
WALTON, EVERETT P., Topsham, Maine.
WASSERMAN, JEANETTE H., Hunter College, New York City.
WHITE, GERTRUDE M., Assistant, University of Wisconsin.
WEINSTEIN, FLORENCE, Student, Barnard College.
WELCH, M. W., 1806 Morse Ave., Chicago, Ill.
WENRICH, MYRA S., Philadelphia, Pa.
YAGLE, ELIZABETH M., Student, Carnegie Institute of Technology.

3. TABULAR VIEW OF ATTENDANCE

	1912	1913	1914	1915	1916
INVESTIGATORS—Total	93	122	129	137	129
Independent:					
Zoölogy	44	58	62	69	70
Physiology	14	17	22	20	23
Botany	10	11	10	6	7
Under Instruction:					
Zoölogy	21	21	31	36	25
Physiology	2	7	1	4	3
Botany	2	7	3	2	1
STUDENTS—Total	67	69	89	105	102
Zoölogy	24	33	43	47	50

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Embryology	15	22	21	37	26
Physiology	11	8	10	15	14
Botany	17	7	15	6	12
TOTAL ATTENDANCE	160	191	218	242	231
INSTITUTIONS REPRESENTED—Total	57	80	77	79	73
By investigators	43	50	51	59	51
By students	36	41	47	42	45
SCHOOLS AND ACADEMIES REPRESENTED:					
By investigators	2	3	1	3	—
By students	1	6	5	9	3

4. SUBSCRIBING INSTITUTIONS—1916

AMHERST COLLEGE
BARNARD COLLEGE
BOWDOIN COLLEGE
BRYN MAWR COLLEGE
CARLETON COLLEGE
CARNEGIE INSTITUTE OF TECHNOLOGY
CLARK UNIVERSITY
COLUMBIA UNIVERSITY
DARTMOUTH COLLEGE
ELSE SERINGHAUS SCHOLARSHIP, HUNTER COLLEGE, NEW YORK CITY
GOUCHER COLLEGE
HARVARD UNIVERSITY
JOHNS HOPKINS UNIVERSITY
KANSAS STATE AGRICULTURAL COLLEGE
KAISER WILHELM INSTITUT FÜR BIOLOGIE
LAKE FOREST COLLEGE
MOUNT HOLYOKE COLLEGE
NORTHWESTERN UNIVERSITY
OBERLIN COLLEGE
PRINCETON UNIVERSITY, DEPT. OF BIOLOGY
PRINCETON UNIVERSITY, DEPT. OF PSYCHOLOGY
RADCLIFFE COLLEGE
ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
RICE INSTITUTE
RUTGERS COLLEGE
SMITH COLLEGE

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H. SOPHIE NEWCOMB MEMORIAL COLLEGE
UNIVERSITY OF CHICAGO
UNIVERSITY OF CINCINNATI
UNIVERSITY OF ILLINOIS
UNIVERSITY OF KANSAS
UNIVERSITY OF MICHIGAN
UNIVERSITY OF PENNSYLVANIA
UNIVERSITY OF ROCHESTER
UNIVERSITY OF TEXAS
UNIVERSITY OF WISCONSIN
VASSAR COLLEGE
WELLESLEY COLLEGE
WISTAR INSTITUTE
YALE UNIVERSITY

5. EVENING LECTURES, 1916

Friday, June 30,
PROF. W. J. V. OSTERHOUT..... "Permeability and Antagonism."

Friday, July 7,
PROF. JACQUES LOEB..... "On Adaptation to Environment."

Tuesday, July 11,
DR. ROBERT M. YERKES..... "The Study of Monkeys and Apes."

Friday, July 14,
DR. JAMES B. MURPHY..... "Studies in Lymphoid Activity."

Tuesday, July 18,
PROF. ALEXANDER PETRUNKEVITCH. "Segmentation in Arthropods."

Friday, July 21,
PROF. J. LAWRENCE HENDERSON.... "Organization as a Physical Chemical Problem."

Tuesday, July 25,
PROF. W. B. CANNON..... "Some Recent Studies of Glands of Internal Secretion."

Friday, July 28,
PROF. RICHARD GOLDSCHMIDT..... "Experimental Intersexuality and the Sex-problem."

Tuesday, Aug. 1,
DR. RAYMOND PEARL..... "Parental Alcoholism and the Progeny in Poultry."

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Tuesday, Aug. 8,

DR. ALFRED G. MAYER ..... "Biological Problems of the Pacific."

MEMBERS OF THE CORPORATION

LIFE MEMBERS

ALLIS, MR. E. P., JR., Palais Carnoles, Menton, France.
ANDREWS, MRS. GWENDOLEN FOULKE, Baltimore, Md.
BILLINGS, MR. R. C., 66 Franklin St., Boston, Mass.
CAREY, MR. ARTHUR ASTOR, Fayerweather St., Boston, Mass.
CLARKE, PROF. S. F., Williamstown, Mass.
CONKLIN, PROF. EDWIN G., Princeton University, Princeton, N. J.
CRANE, MR. C. R., Woods Hole, Mass.
DAVIS, MAJOR HENRY M., Syracuse, N. Y.
EVANS, MRS. GLENDOWER, 12 Otis Place, Boston, Mass.
FARLOW, PROF. W. G., Harvard University, Cambridge, Mass.
FAY, MISS S. B., 88 Mt. Vernon St., Boston, Mass.
FOLSOM, MISS AMY, 88 Marlboro St., Boston, Mass.
FOOT, MISS KATHERINE, 955 Park Ave., New York City, N. Y.
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STUDIES ON THE BIOLOGY OF PARACOPIDOSOMOPSIS II. SPERMATOGENESIS OF MALES REARED FROM UNFERTILIZED EGGS.[1]

J. T. PATTERSON AND LELIA T. PORTER.

In the first paper of this series it was pointed out that the unfertilized egg of *Paracopidosomopsis floridanus* produces a polyembryonic brood of males. It is the purpose of this paper to show that the germ cells of such males are characterized by the haploid number of chromosomes. As in many other Hymenoptera, the presence of the half number is due to the fact that in the parthenogenetic development of the matured egg there is no compensatory process for restoring the full number. As a consequence of the reduced number of chromosomes in these males, the maturation divisions are modified in such a way that two, instead of four, spermatozoa are produced from each first spermatocyte. In the somatic cells of these males the haploid number, subject to certain variations, also prevails; but this is a subject that will be considered later.

Material and Methods.

All of the material used in the study of spermatogenesis has been taken from animals reared under experimental conditions. It consists of two large broods of larvæ and a series of one hundred pupæ taken from a mummified carcass of the host. The two larval broods were preserved forty-eight and twenty-four hours, respectively, prior to the time at which pupation would have occurred. The series of pupa stages were preserved at close intervals, beginning twenty-four hours after the formation of the carcass.

The three broods yielding this material resulted from an experiment designed to determine whether the unfertilized egg

[1] Contributions from the Zoölogical Laboratory of the University of Texas, No. 131.

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developed into a brood of males. A summary of the experiment is as follows:

Host eggs laid the night of November 7.

Each egg parasitized by one oviposition of an unfertilized female, 2:30 P.M., Nov. 8.

Caterpillars hatched Nov. 13.

First brood of parasites preserved Dec. 4.

Second brood of parasites preserved Dec. 5.

Carcass of third caterpillar formed Dec. 6.

Series of pupa stages from carcass preserved Dec. 7–19.

Brood of 1842 male parasites emerged Dec. 25.

We have used a great variety of fixing fluids on polyembryonic material, but for the cytology of male germ cells no reagent has been found that equals Bouin's picro-formol-acetic mixture to which crystals of urea have been added. Preparations of the gonads fixed in this fluid and treated with Heidenhain's iron hæmatoxylin, counterstained with Orange G, give very fine results. However, this method of fixation has the disadvantage of not being adapted to the staining technique usually employed to bring out certain cytoplasmic structures, such as the mitochondria. Nevertheless, we have used it almost exclusively because of our especial interest in the chromosomes.

Even with the use of the best methods of technique, Hymenoptera material is never as favorable for the study of chromosomes as that of many other insects, notably the Hemiptera and Orthoptera. In certain respects, the germ cells of *Paracopidosomopsis* are more favorable for cytological study than those of many other species of the order, but even in this species the chromosomes are, at certain stages, greatly elongated, with a consequent tendency to become entangled with one another. This often makes difficult the determination of the exact number. The difficulty is somewhat increased by the smallness of the cell, which at the end of the growth period does not exceed a diameter of seven or eight microns. The best counts of chromosome numbers are obtained in somatic cells, because in these cells the metaphase plate is flat and the chromosomes are more scattered than in the spermatocytes. In any event, one does not experience difficulty in distinguishing a metaphase plate

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with the haploid number of chromosomes from one having the diploid number.

The Spermatogonia.

We have not attempted to follow closely the course of development of the male gonads in the larva. At the earliest stage at which the larva can be recognized as a male, each testis consists of a solid spherical mass of cells. Histologically, there are two kinds of cellular elements, germ cells and epithelial cells. During the course of further development the gonad elongates, and at the same time cyst formation occurs. This process is completed by the end of the larval period.

At no time during the development of the gonad does one find any great number of spermatogonial divisions. Four or five mitotic figures are the most that will be found at any one time in a given testis.

The period of multiplication ends sometime between forty-eight and twenty-four hours prior to pupation. Of the two larval broods mentioned above, the one preserved forty-eight hours before the time of pupation has a few larvæ showing spermatogonial divisions, while the one fixed twenty-four hours later has no divisions.

Fig. 1 shows a metaphase plate of one of the dividing spermatogonia. The plate has eight elongated chromosomes, one of which shows the beginning of a longitudinal split. We have concluded from a study of all of our material of both males and females that eight represents the haploid number of chromosomes.

The Growth Period.

The growth period begins shortly after the cessation of the spermatogonial divisions, and extends through the fourth day after pupation. At the beginning of this period all of the germ cells in a given cyst are in the same stage of development. In section each cell appears wedge-shaped, with its slightly curved base lying against the outer membrane and its apex directed toward the center of the cyst. The apexes of all the cells in the cyst are usually connected by a common mass, which is apparently the remains of the interzonal fibers of the last spermatogonial division.

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The growth stage is characterized by the presence of a conspicuous nucleolus, which occupies an excentric position in the nucleus (Figs. 2–4). At first the nucleolus is somewhat diffuse, but later its outline becomes sharp and distinct (Fig. 2). It frequently has several less deeply stained areas lying at its center (Fig. 3). We have good reasons for believing that the nucleolus is chromatin in character.

One of the most striking changes to occur in the cell during growth, is the appearance of certain cytoplasmic inclusions, which stain intensely black with iron hæmatoxylin. These are first seen about seventy-two hours after pupation, when they appear as dark, elongated areas in the cytoplasm. Later, as the cell enlarges, the areas become more definite and appear as seen in Fig. 3. These structures soon disappear (Figs. 4, 5), although some of them may persist until after the first maturation division is well advanced (Figs. 6, 8). We have not attempted to determine the origin and nature of these cytoplasmic inclusions; but, judging from the work of Meves on the bee, they are probably mitochondrial in character.

The presence of these inclusions greatly interferes with the study of the changes which now take place in the cell. Especially is this true with reference to those changes involving the behavior of the centrosomes. We shall therefore confine our account to the changes in shape of the cell which occur during the growth period. As already stated, all of the cells of a cyst are at first united at their pointed ends by the interzonal connections. In some cells these connections are soon lost, and each cell then becomes polyhedral in outline (Figs. 2, 3), and finally spherical. In other cells the interzonal connections apparently persist throughout the entire growth period, and after these are finally severed, the pointed end of each primary spermatocyte contains the remains of the original interzonal connection in the form of an "interzonal body" (Mark and Copeland).

At the end of the growth period many cells are met with which possess a delicate process. It is difficult to demonstrate the presence of a centrosome at the end of this process (Fig. 4). With reference to the position of the cell in the cyst, the process may lie on any side of the cell. It is not clear whether such a

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process is formed in every cell. If it is of constant occurrence, the period of its existence must be very brief; for at the beginning of the first spermatocyte division, which soon follows, it is entirely absent.

A similar process has been described for the young spermatocytes of several different Hymenoptera, but the most careful and detailed work on it is that of Meves ('07) on the honey bee. According to Meves, the process in the bee is formed through the influence of one of the primary centrosomes ("Hauptcentriolen"), which arise by division from the original centrosome of the cell. The process with its centrosome is soon withdrawn into the cell. One of the several secondary centrosomes ("Nebencentriolen") which had previously arisen by division from the primary centrosomes, then participates in the formation of the mitotic figure responsible for the production of the so-called polar body.

While it is not possible in our material to demonstrate all of these fine points, yet it is highly probable that the cytoplasmic process in the germ cells of *Paracopidosomopsis* has a similar history to that described by Meves for the germ cells of the bee.

First Maturation Division.

Preparation for the first maturation division is made manifest by changes in the nucleus. It is also made evident by changes in the shape of the cell, especially in the spermatocytes which have early lost their interzonal connections. In cells in which these connections persist up until the end of the growth period, the so-called polar body, or rudimentary second spermatocyte, is budded off from the side of the cell that had recently had the connection. In the other spermatocytes a blunt protrusion arises at one side (Fig. 5), which soon develops into a pointed process (Fig. 6). At the pointed end thus formed there is present a tiny centrosome, from which radiate a number of delicate fibers toward the nucleus. At the opposite, or blunt end of the cell, a similar centrosome with its fibers is also present.

The most important change in the nucleus concerns the nucleolus, which during the first half of the growth period remains a deeply staining, spherical mass (Figs. 2, 3). A number

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of spherical granules arise at its periphery, giving it the appearance of a lobulated structure (Fig. 4). Eventually the nucleolus breaks up into a number of these large granules, which lie in a finely granular matrix (Fig. 5). Apparently these larger granules coalesce to form the chromosomes (Fig. 7). From a careful study of this particular stage, one can not escape the conclusion that the chromosomes arise from the nucleolus.

A somewhat similar method of origin of chromosomes has been described by Meves and Duesberg ('08) in the male germ cells of *Vespa crabro*. They say: "Das Herannalien der ersten Reifungsteilung macht sich dadurch bemerkbar, das im Kern in der Umgebung des grösseren Nucleolus immer mehr Chromatinkörner auftreten. Der Nukleolus selbst wird dabei immer kleiner. Man gewinnt den Eindruck, dass seine Substanz in diejenige der Chromatinkörner übergeht." There is one important difference between the two forms. In *Paracopidosomopsis* the nucleolus gradually but completely disappears as the chromosomes are formed, while in *Vespa* a small body still remains after the chromosomes are organized.

During the organization of the chromosomes the nucleus elongates in the direction of the long axis of the cell. An imperfect intranuclear spindle then arises, and upon this the chromosomes tend to take up an equatorial position (Figs. 6, 8). They are usually so closely massed together that it is impossible to make an exact determination of their number. Fig. 6 shows a very interesting case, in which a single curved chromosome has moved to the upper end of the nucleus, where it lies in contact with the inner surface of the nuclear membrane. On account of the massed condition of the chromosomes, it is not possible to tell whether this particular chromosome is the product of a recent division, and has a sister chromosome lying within the mass. However, this is an isolated case, and it is clear that the chromosomes do not normally divide in the first spermatocyte division. Instead, only the cytoplasm undergoes division, and this in a very adequal manner (Figs. 7, 8).

The constriction of the cytoplasm begins about one-third the distance from the pointed end of the cell (Fig. 7) and finally results in cutting off a small, knob-like mass entirely free of

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chromatin (Fig. 9). This abortive division is homologous to the first spermatocyte division which normally occurs in other forms, and the two unequal cells thus produced are to be regarded as equivalent to second spermatocytes.

The small non-nucleated cell, or mass of cytoplasm, soon degenerates, although in some cases it may persist until the second maturation division is well advanced. All stages in the disintegration of the mass are to be seen in a single cyst. The fragmenting masses lie in the interstices of the dividing second spermatocytes.

The most interesting point to be noted in connection with this abortive maturation division is the fact that nothing comparable to a synapsis of chromosomes occurs. The absence of this phenomenon, which is universal in the spermatogenesis of males arising from fertilized eggs, is undoubtedly due to the fact that the unmatured, male germ cell of *Paracopidosomopsis* possesses but the haploid number of chromosomes. The function of the first maturation division is to bring about a reduction from the diploid to the haploid number of chromosomes; but the males of this species, by virtue of their origin from matured, but unfertilized eggs, already possess the half number, and consequently, the first maturation results in a feeble or abortive attempt at a division.

Second Maturation Division.

Throughout the entire period occupied by the first maturation, the nuclear membrane remains intact (Figs. 5–9). At the end of this period the incomplete intranuclear spindle disappears, as do also the fibers which radiate from the centrosome included in the small cytoplasmic mass (Fig. 9). On the contrary, the aster, which lies at the lower side of the nucleus in the true second spermatocyte, persists. It is highly probable that this aster, by a division of its centrosome, gives rise to the second maturation spindle, as is the case in the European hornet.

In the brief interim between the first and second maturations, the nucleus enters a rest stage. The chromatin recedes to that side of the nucleus lying farthest from the cytoplasmic bud, and becomes so massed that it is impossible longer to distinguish

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clearly individual chromosomes (Fig. 9). In the stages immediately following this one, every phase in the reorganization of the chromosomes is clearly demonstrable. In having a definite resting stage between the abortive and the true division, *Paracopidosomopsis* resembles *Neuroterus* (Doncaster, '09), but differs from such forms as the bee and the wasp.

After the cytoplasmic bud is cut off, the second spermatocyte becomes spherical in outline, and a conspicuous maturation spindle is formed (Fig. 12). The reorganized chromosomes are drawn into the equatorial position on this spindle. Clear polar views are difficult to find, owing to the fact that the chromosomes are elongated and frequently twisted (Fig. 12). In the clearest cases observed, the number of chromosomes is seen to be eight (Figs. 10, 11).

The axis of the second maturation spindle bears no definite relation to that of the first maturation spindle. This was determined by studying those second spermatocytes to which the cytoplasmic bud remains for some time slightly attached. It was found that the axis of the second spindle has no definite relation to the point of attachment.

In the second division the chromosomes split lengthwise, and the daughter chromosomes pass to the opposite poles of the spindle (Fig. 14). It is frequently easy to count the eight daughter chromosomes passing to the one or the other of the poles, but cells in which both daughter groups can be counted are not often met with (Fig. 15). We conclude from these observations that all of the chromosomes divide in the second maturation. While in the anaphases the chromosomes are often well scattered, yet we have found no evidence of a distinctly "advancing" or "lagging" member, such as might indicate that a particular chromosome had failed to divide. From this it follows that the two spermatids resulting from the second division will be alike. That is to say, the spermatozoa in *Paracopidosomopsis* will not be dimorphic.

In passing to the poles of the spindle the chromosomes keep their long axes parallel to the long axis of the spindle (Figs. 15, 16), and upon reaching the pole fuse to form a solid, deeply staining mass of chromatin out of which the spermatid nucleus arises (Figs. 17, 18).

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After the division of the cytoplasm, the two spermatids remain connected for some time by means of the interzonal fibers (Fig. 18). After they separate, each differentiates into a typical spermatozoön (Figs. 19, 20). We shall not discuss further the subject of the metamorphosis of the spermatids, except to emphasize the point that both spermatids must form functional germ cells, as we have found no evidence of degenerating spermatids or spermatozoa.

Comparison with other Hymenoptera.

Studies on the spermatogenesis of Hymenoptera have shown that there is a striking modification of the maturation process in those species in which sex-determination is supposed to be in accordance with the Dzierzon theory. Instead of four spermatozoa arising from each first spermatocyte, only one or two matured germ cells develop. In one group of these Hymenoptera both spermatocyte divisions are abortive; the first division cutting off a small cytoplasmic bud free of chromatin, and the second division a small bud which receives half the chromatin. There is thus produced but a single spermatid, and consequently only one spermatozoön. In the second group the first division is likewise abortive, producing a cytoplasmic bud, but the second division is equal, producing two similar spermatids, which metamorphose into spermatozoa.

To the first of these groups belongs the honey bee, as has been shown by the work of Meves ('03, '07), Mark and Copeland ('06), and Doncaster ('06, '07). To this class there also probably belongs the solitary bee (*Osmia cornuta*) worked on by Armbruster ('13).

The second group includes a number of different species, of which the following may be mentioned: *Xylocopa violacea* (Granata, '10, '13), *Neuroterus lenticularis* (Doncaster, '09), *Vespa crabro* (Meves and Duesberg, '08), *Vespa maculata* (Mark and Copeland, '07), *Vespa germanica* (Meves, '03), *Camponotus herculaneus* (Meves and Duesberg, '08, and Lams, '08), *Dryophanta erinacei* (Wieman, '15). *Paracopidosomopsis floridanus* also clearly belongs to this class.

While the various investigators report differences as to details

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in the two types of spermatogenesis outlined above, yet there is great uniformity in their interpretations of the general character of the processes involved. Our own work on *Paracopidosomopsis* indicates that the spermatogenesis of this species is very similar, not only as regards the general character of the process, but also as regards many details, to the maturations of the European hornet, *Vespa crabro*, as shown by the work of Meves and Duesberg.

AUSTIN, TEXAS,
February 6, 1917.

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DESCRIPTION OF FIGURES.

All of the figures are camera drawings made at table level, with the Zeiss 2 mm. lens and No. 12 compensating ocular. The figures thus outlined were enlarged three times by the aid of an enlarging camera, and then reproduced with one third off. They are therefore magnified about 3857.

PLATE I.

FIG. 1. Metaphase plate of last spermatogonial division.

FIG. 2. Stage at the beginning of growt' period.

FIG. 3. Stage at the mid-growth period, showing the conspicuous cytoplasmic inclusions.

FIG. 4. Spermatocyte with delicate cytoplasmic process.

FIG. 5. Elongation of the cell in preparation for the first spermatocyte division.

FIG. 6. Stage showing the imperfect intranuclear spindle.

FIG. 7. Stage showing the beginning of the constriction which results in cutting off the cytoplasmic bud.

FIG. 8. A later stage showing the same process.

FIG. 9. Stage showing the end of the first maturation division. The cytoplasmic bud has just been cut off.

FIG. 10. Metaphase plate of the second spermatocyte.

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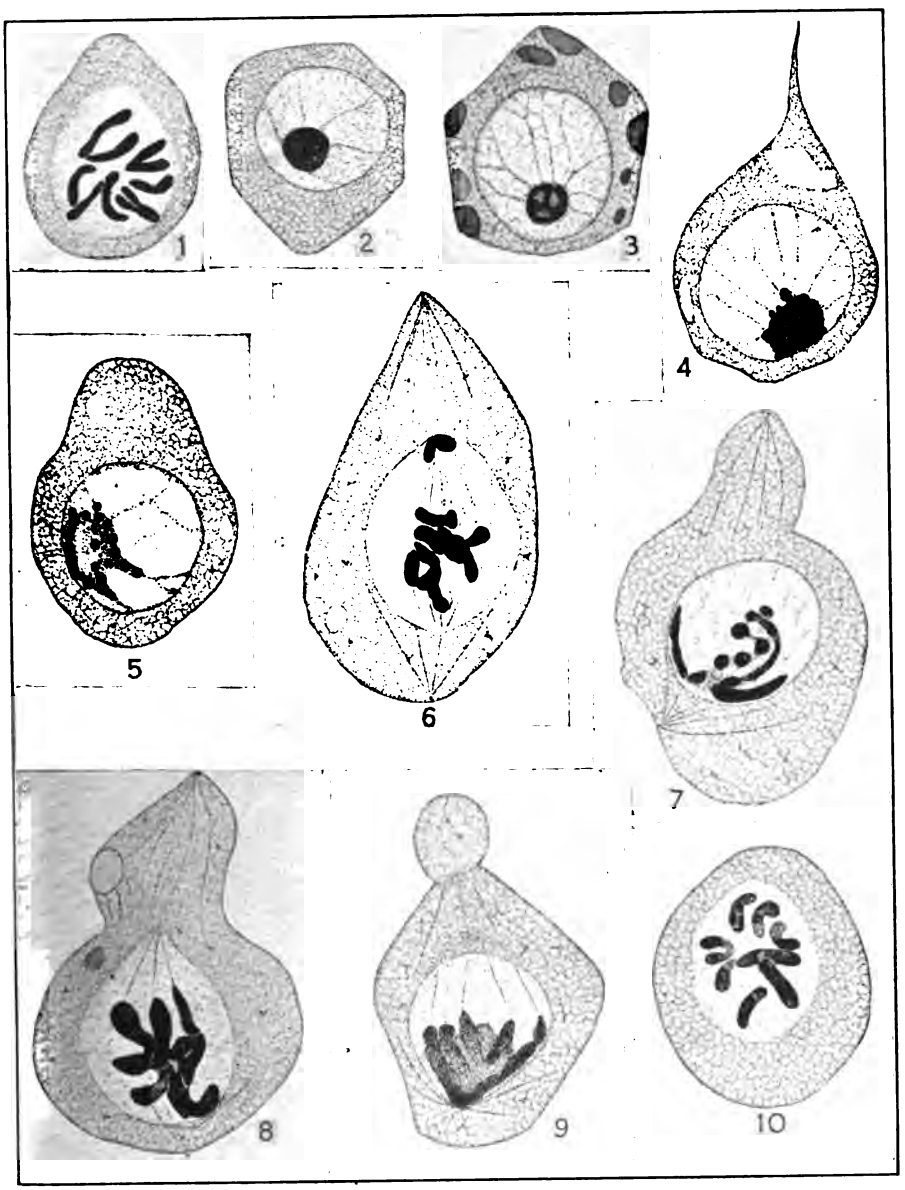

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PLATE II.

FIG. 11. Metaphase plate of the second spermatocyte.

FIG. 12. Side view of the second maturation spindle, showing four twisted chromosomes.

FIG. 13. A section through one side of the spindle, showing two chromosomes that have just divided. Parts of two other chromosomes are also present.

FIG. 14. A similar section, showing the complete division of four chromosomes.

FIG. 15. Anaphase stage, showing eight daughter chromosomes passing to each pole.

FIG. 16. Late anaphase stage.

FIG. 17. Telophase stage.

FIG. 18. The two spermatids, still connected by means of the interzonal fibres.

FIG. 19. A completely separated spermatid.

FIG. 20. A later stage of the spermatid.

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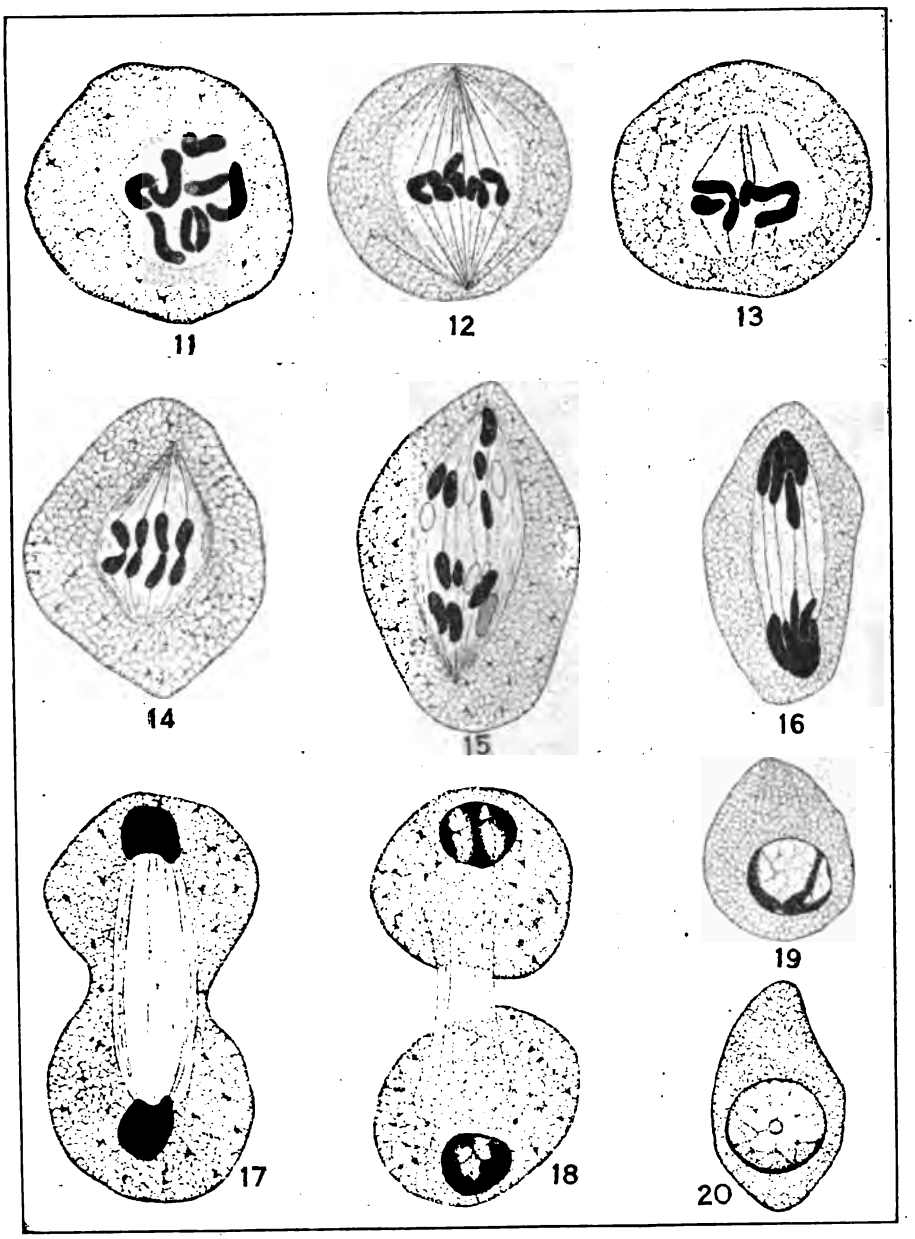

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RHYTHMS AND ENDOMIXIS IN VARIOUS RACES OF PARAMÆCIUM AURELIA.

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OSBORN ZOÖLOGICAL LABORATORY, YALE UNIVERSITY.

In view of the fact that the study of rhythms and endomixis in *Paramœcium* was made chiefly on my long pedigreed race of *Paramœcium aurelia*, it has been suggested by a couple of authors that the reorganization process may be something peculiar to this race, perhaps resulting from long subjection to conditions which preclude the possibility of conjugation. This assumption, however, was rendered exceedingly improbable from the data presented by Woodruff and Erdmann[1] in their original complete paper which showed that "the same reorganization process was resumed in all the lines within a relatively short time after conjugation" was allowed to occur in a subculture of the main race, and also that the phenomenon was observed early in the culture of a race which was secured by Erdmann from Berlin, Germany. Further, Erdmann and Woodruff[2] in a paper on endomixis in *Paramœcium caudatum* stated incidentally that endomixis had been found in two new races of *Paramœcium aurelia* immediately upon their isolation.

The above data more than justify the early conclusion of Woodruff and Erdmann that "this reorganization process is a normal phenomenon and *probably occurs in all races of the species Paramœcium aurelia*,"[3] but in connection with other work on *Paramœcium* it has been necessary to employ various races for study and the purpose of the present paper is to record briefly the additional data bearing on the points under discussion. This may be most readily accomplished by considering seratim the graphs of the life histories which show the rhythms and the occurrence of endomixis in the respective cultures. Since

[1] *Journal of Experimental Zoology*, Vol. 17, No. 4, 1914.

[2] *Journal of Experimental Zoology*, Vol. 20, No. 2, 1916.

[3] *Ibid.*, 1914, p. 474.

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the methods of conducting the cultures, plotting the graphs, etc., has been fully described in earlier papers from this laboratory, the reader is referred to them for details of technique. It is only necessary to emphasize here that the occurrence of endo-

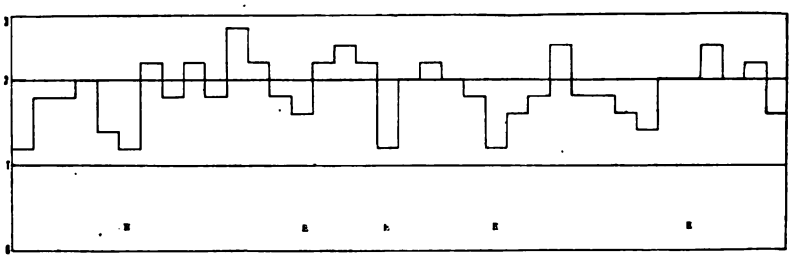

FIG. 1.

mixis has been determined, of course, in each case by the cytological study of animals preserved almost daily from each culture during the periods under discussion.

Race I. (*Woodruff's Main Culture.*)—Isolated May 1, 1907, at Williamstown, Mass., and carried in pedigreed culture to

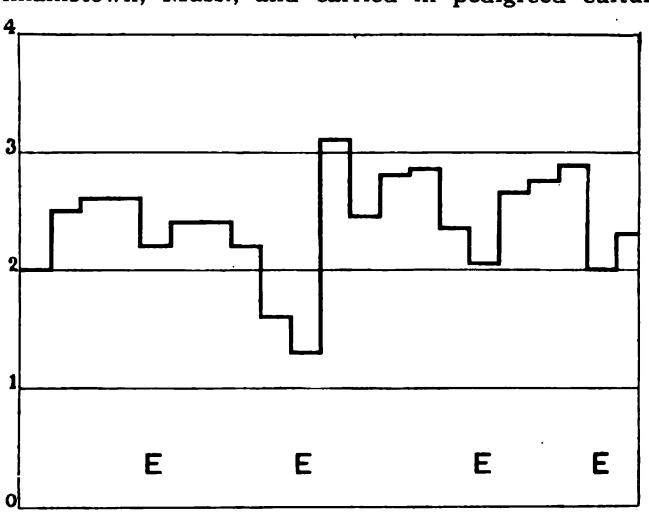

FIG. 2.

date (July, 1917). During this period of more than ten years it has attained over 6,000 generations without conjugation. The discovery of endomixis was made on this race and full details

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have been presented in earlier papers. Fig. 1[1] gives a typical graph of a subculture showing the relation of rhythms and endomixis.

Race II. (*Berlin Race.*)—Isolated by Erdmann in Berlin. For details see Woodruff and Erdmann, 1914.

Race III. (*Oberlin Race.*)—The initial animals of this pedigreed culture was supplied by Professor R. A. Budington, of Oberlin, Ohio. The culture was started October 8, 1914, and was discontinued after nearly two years during which time over 1,000 generations were obtained. Fig. 2 is a graph of the average division rate of the four lines of this culture again averaged for each of the first 21 five-day periods of its life when it was being intensively studied with reference to the occurrence of rhythms and endomixis. The graph shows four rhythms and at the low point between each endomixis occurred. It is to be

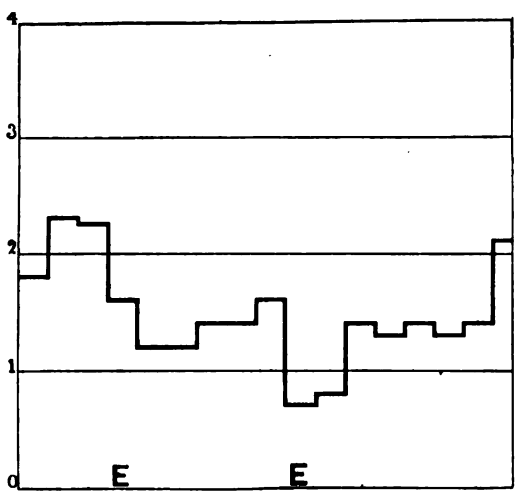

FIG. 3.

noted that the reorganization process appeared during the fifth five-day period after the isolation of the culture, thus showing once more that this phenomenon is not dependent upon long subjection to culture conditions for its genesis. The length of the rhythmic periods for this race is also essentially the same as in Race I (cf. Fig. 1).

Race IV. (*Bryn Mawr Race.*)—The animal which started

[1] *Ibid.*, 1914, Fig. 17.

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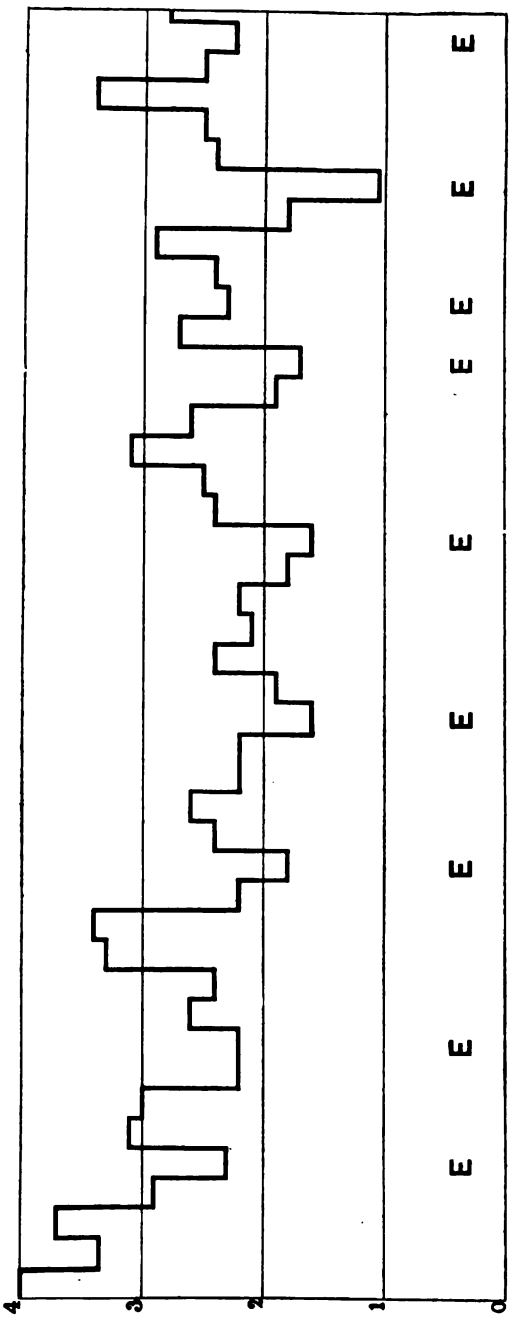

FIG. 4.

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this pedigreed culture was isolated on January 7, 1915, from material supplied by Dr. Florence Peebles from Bryn Mawr, Pa. The culture was discontinued at the 650th generation on February 8, 1916. Fig. 3 shows the relationship of rhythms and endomixis during the first eighty-five days of the culture when the animals were being examined for the reorganization process. The results fully substantiate those obtained from previous races. The last inter-rhythm period shown in the diagram is apparently longer than usual—though it is possible that endomixis was overlooked about the 15th five-day period.

Race V. (*Oxford Race.*)—Culture started, from material secured at Oxford, Ohio, on July 16, 1915, and discontinued on August 7, 1916, at the 1,000th generation. The division rate of the culture is shown in Fig. 4 for the first 44 five-day periods of

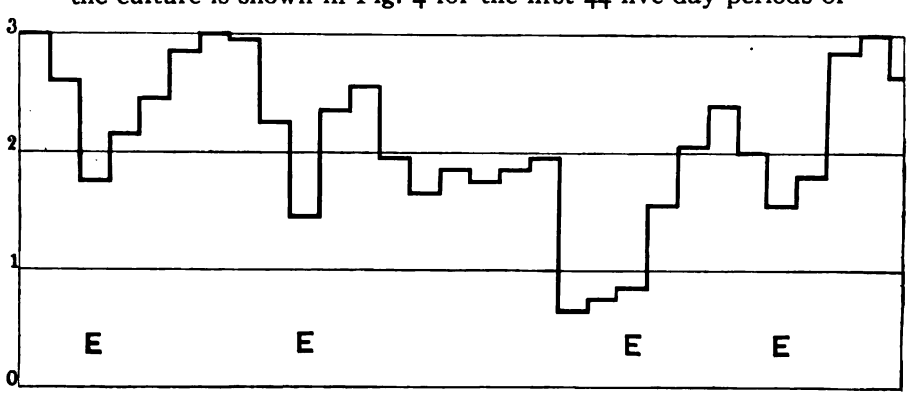

FIG. 5.

its life. During this time nine rhythms occurred at each of which endomixis was observed. It is hoped that readers who may possess doubts concerning rhythms and endomixis will carefully consider this graph.

Race VI. (*Woods Hole Race.*)—This race was obtained by Dr. G. A. Baitsell at Woods Hole, Mass., and its pedigreed culture was begun on August 11, 1915, and discontinued on January 14, 1916, at the 305th generation. Fig. 5 gives the graph of the division rate of this culture throughout its existence.[1]

[1] The division rate is plotted consistently one quarter of a division per day too fast for every five-day period, owing to a draughtsman's error. An *E* should be inserted in the first period.

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Endomixis occurred as soon as the culture was started and recurred at the usual intervals. The animal was not studied cytologically during the 14th five-day period so that endomixis is not indicated by an *E* in this period on the chart, but from the character of the curve there can be little doubt that an examination of stained specimens would have revealed it.

In a word, then, the data from every culture of *Paramœcium aurelia* (isolated from as diverse localities as Ohio and Germany) which we have studied prove beyond doubt, I believe, that endomixis is a normal periodic phenomenon which occurs in all races of *Paramœcium aurelia*.

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BIOLOGICAL BULLETIN

STUDIES ON THE BIOLOGY OF PARACOPIDOSOMOPSIS.

III. MATURATION AND FERTILIZATION.[1]

J. T. PATTERSON.

INTRODUCTION.

In the second paper in this series (Patterson and Porter, '17) an account of the spermatogenesis was given. It was shown that males reared from unfertilized eggs carry the haploid number of chromosomes in their germ cells, and that as a consequence the first maturation division is abortive, while the second results in producing two equal spermatids, and hence two similar spermatozoa. The purpose of the present paper is to give an account of the maturation of the egg, and of fertilization.

It was originally planned to report on the maturation process both in fertilized and unfertilized eggs, but my series of the latter has proved to be very incomplete, so that the present account is based entirely on a study of inseminated eggs. However, it is highly probable that maturation is identical in the two types of eggs of *Paracopidosomopsis*, as Silvestri ('06) has found to be the case in *Litomastix*.

It is not a difficult matter to obtain material for the study of maturation. The best way is to place a few non-parasitized moth eggs in a closed vessel containing a large number of female parasites. In the course of an hour each host will have deposited in it several eggs of the parasite—sometimes as many as twenty-five or thirty. The host eggs, thus serving as containers for the parasite eggs, can then be fixed as desired. No difficulty has

[1] Contribution from the Zoölogical Laboratory of the University of Texas, No. 132.

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been experienced in cutting the moth eggs into sections from three to five microns thick. Preparations of the entire egg have also been used in the study of maturation. These were made from smears of the host egg.

The Freshly Deposited Egg.

The freshly laid egg of *Paracopidosomopsis* is a pear-shaped cell, very similar in appearance to that of *Litomastix*, as figured and described by Silvestri ('06). Different eggs vary considerably in size, and to some extent also in shape. In Fig. 2 is shown a typical egg some time after it had been deposited. The average egg measures about 115 μ in its long axis and 60 μ at its widest diameter.

The egg may be divided into two parts, the anterior portion or pointed end, and the posterior portion or broad end. At the time of deposition there is no line of demarcation between these two parts, but after the beginning of cleavage they are recognized as the polar region containing the polar nuclei, and the embryonal region consisting of the true embryonic cells.

The egg is surrounded by a thin but tough membrane, probably a true chorion. It is also possible that a very delicate vitelline membrane is present, although this is difficult of demonstration.

The contents of the egg consists of a very finely granular protoplasm in which are found a few scattered yolk or oil spherules (Fig. 8). The fertilized egg contains three distinct bodies, the germinal vesicle, the so-called nucleolus, and the sperm (Fig. 1).

The germinal vesicle is a very conspicuous spherical body, mesuring about 19 μ in diameter. Its chromatin is in the form of small stellate bodies. There is also present a small but distinct nucleolar-like body (Fig. 1, *n*). Although the position of the nucleus, as well as that of the nucleolus and of the sperm, is very variable, yet it usually lies toward the pointed end of the cell.

The so-called nucleolus in the eggs of polyembryonic insects has given rise to a great deal of discussion as to its origin and fate. Silvestri ('06, '08), who first described and named this body, thought that it arose at an early stage of the oöcyte from the germinal vesicle, but Martin ('14) and Hegner ('14, '15)

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have demonstrated that it arises outside the germinal vesicle, and therefore does not represent a true nucleolus. Silvestri ('14) has later admitted the correctness of their conclusions, and proposed the term "oösoma." As to the fate of this body, Silvestri showed that it enters into certain definite cleavage cells, which he believed give rise to germ cells. Hegner has elaborated this idea, bringing it into harmony with his well-known views on germ-line-determinants.

The sperm is invariably found in the posterior half of the egg (Figs. 1, 2, 8), indicating that insemination must take place at any point on the surface of the broad end of the egg. The entire sperm enters the egg (Fig. 8), and soon thereafter forms a small male pronucleus (Figs. 1, 2, 13). There is no evidence that polyspermy ever occurs in the egg of this species, as no egg has ever been found with more than a single sperm in it.

First Maturation.

As in the eggs of many other Hymenoptera, the maturation divisions of *Paracopidosomopsis* do not result in the formation of distinct polar bodies, but instead only the chromatin undergoes division. Preparation for the first maturation division is seen in the appearance of heterotypic or bivalent chromosomes in the early prophase stage. These are sometimes in the form of V's and crosses, and in the clearest cases eight are present (Fig. 7).

The position of the first maturation spindle is very constant. It lies well toward the anterior end of the cell, with its long axis directed toward the center of the egg (Fig. 8). In the anaphase stage there is present a distinct mid-body at the equator of the interzonal or spindle fibers (Figs. 8, 9). At the close of the division the two groups of chromosomes are not reorganized into nuclei; but lie close together surrounded by clear spaces in the cytoplasm.

In only a few cases have I been able to determine accurately the number of chromosomes that pass to either end of the first maturation spindle. In Fig. 9 the outer, or first polar body group contains eight chromosomes, and at the lower end at least seven can be counted. In Fig. 10 the lower or second oöcyte group

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has eight clear chromosomes. In the upper or first polar body group eight are also present, but they are somewhat massed together. These observations indicate that the first maturation division results in separating the two components of each bivalent chromosome, so that the first polar body receives eight whole chromosomes, as does likewise the second oöcyte.

Second Maturation.

The second maturation occurs soon after the first is completed. The chromatin does not reorganize a nucleus. The first polar body chromatin likewise divides without forming a nucleus.

The second maturation and the division of the polar body may occur simultaneously (Fig. 12), or the polar body division may either precede (Fig. 11) or follow (Fig. 2) the second maturation division. There is therefore no close correlation between the divisions of these two groups of chromosomes.

In Fig. 11 the late anaphase stage of the first polar body division shows eight chromosomes at each end of the spindle. The group of chromosomes lying just below also has eight. This is the second oöcyte group. In Fig. 12 the two division figures are in the late anaphase stage. At the upper end of the polar body spindle (above) six chromatin masses are present, while at the lower end there are eight. The second oöcyte spindle has seven above and eight below. If these two divisions are equational in character, we should expect to find eight chromosomes in each group. Our failure to do so is probably due to the fact that one or more chromosomes in a group may be hidden by some of the other chromosomes.

Fertilization.

As already stated the egg is inseminated by a single sperm, which lies in the lower half of the newly laid egg. Although the entire sperm enters the egg, only the sperm head becomes transformed into the male pronucleus, the formation of which is completed by the close of maturation.

Of the four groups of chromosomes referred to in connection with Fig. 12, the three lying nearest to the surface of the egg represent the three polar bodies, while the one lying toward the

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center is the oötid group. The fate of the polar bodies will be considered later. The oötid group soon forms a small, condensed female pronucleus, which approaches the male pronucleus with which it eventually fuses. The pronuclei come together in the lower half of the egg. Apparently for a considerable period they lie close together before the actual fusion occurs. In Fig. 13 the two condensed pronuclei lie a short distance apart, with a small aster and its tiny centrosome lying between them. This case is interesting because it is the only one in which I have observed either a centrosome or aster in the eggs of this species.

The pronuclei remain apart while they undergo expansion, the female nucleus always being somewhat larger than the male nucleus (Fig. 15). Upon coming in contact with each other, the pronuclei fuse (Fig. 14) to form a single large conjugated, or cleavage nucleus (Fig. 16). Many different stages of conjugating nuclei have been observed in the preparations, one slide alone showing more than a dozen cases.

Discussion.

These observations show that in the egg of *Paracopidosomopsis* two typical maturation divisions occur, resulting in reducing the sixteen chromosomes of the primary oöcyte to eight in the oötid. It was shown in the second paper of the series that the sperm also receives eight chromosomes. The fertilized egg should therefore have sixteen chromosomes. A study of cleavage divisions shows that this is the case. Fig. 17 is a polar view of a metaphase plate of the first cleavage spindle. It has sixteen chromosomes. In Fig. 18 a side view of an anaphase stage is shown. This is one of the first four blastomeres undergoing division. There are fifteen chromosomes lying toward each end of the spindle, and the sixteenth, just completing its division, lies at the middle of the spindle, on the right.

A study of somatic cells shows that the diploid number of chromosomes is also present in them. Thus the dividing cells of the central nervous system of female larvæ have sixteen chromosomes (Figs. 3, 4). On the other hand, dividing cells from the central nervous system of male larvæ have but eight chromosomes (Figs.

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5, 6). This is what would be expected, if males develop parthenogenetically from eggs that have undergone maturation.

AUSTIN, TEXAS,
March 5, 1917.

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DESCRIPTION OF FIGURES.

PLATE I.

FIG. 1. Entire egg from a smear preparation, made 23 minutes after oviposition. It shows the germinal vesicle (*n*), the sperm (*s*) and the nucleolus (*no*). ×720.

FIG. 2. Entire egg from a smear preparation, showing sperm (*s*), 1st polar body undergoing division (*1 p.b.*), second polar body (*2 p.b.*), egg nucleus (*n*), and nucleolus (*no*). ×737.

FIG. 3. Metaphase plate of dividing cell from central nervous system of female larva. It shows 16 chromosomes. ×3857.

FIG. 4. Same as 3. ×3857.

FIG. 5. Metaphase plate of dividing cell from central nervous system of male larva. It shows 8 chromosomes. ×3857.

FIG. 6. Same as 5. ×3857.

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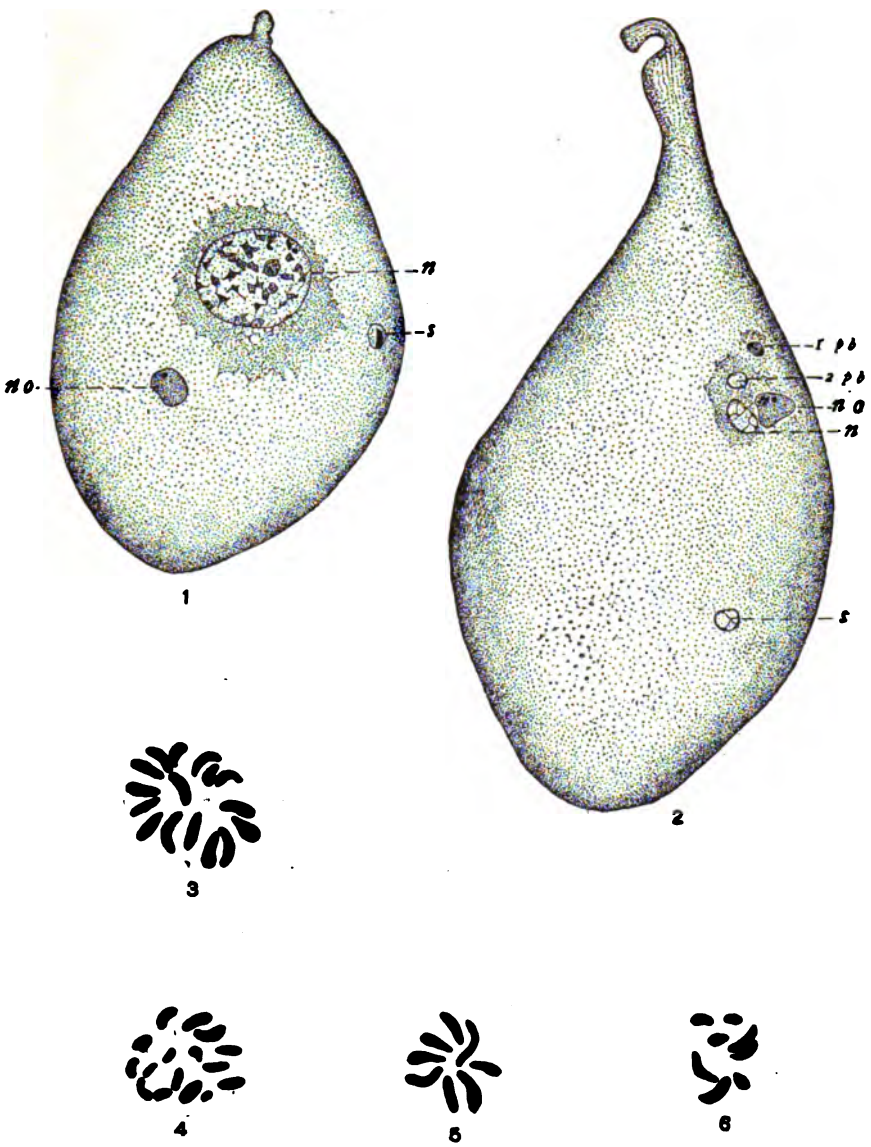

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PLATE II.

FIG. 7. Early prophase of first maturation. The black body close to germinal vesicle is the nucleolus. ×1,240.

FIG. 8. Late anaphase of first maturation. The sperm was drawn in from the next section to the left in the series. ×1,240.

FIG. 9. First maturation spindle. ×1,827.

FIG. 10. First polar body chromosomes above, and chromosomes of the second oöcyte below. ×1,827.

FIG. 11. Anaphase stage of first polar body above, and chromosomes of second oöcyte below. ×1,827.

FIG. 12. Anaphase stages of the first polar body above, and second maturation below. ×1,240.

FIG. 13. Very early stage of the pronuclei, female above, male below. Note tiny aster lying between them. ×1,240.

FIG. 14. Fusion of male and female pronuclei. ×1,240.

FIG. 15. Pronuclei, male above, female below. ×1,240.

FIG. 16. Conjugated nucleus. ×1,240.

FIG. 17. Polar view of first cleavage spindle, showing sixteen chromosomes. ×1,827.

FIG. 18. Side view of cleavage spindle of one of the first four cells. There are fifteen chromosomes in each daughter group. The sixteenth chromosome, which has just divided, lies on right side of spindle. ×1,827.

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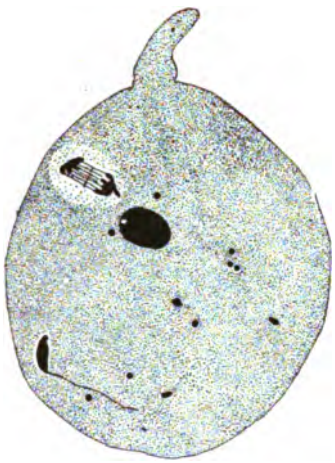
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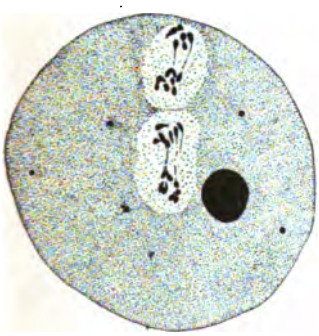
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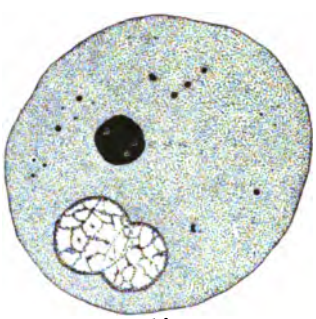
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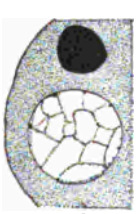
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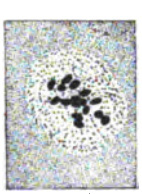
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SOME CHEMOTROPIC AND FEEDING REACTIONS OF PLANARIA MACULATA.

ROSALIND WULZEN.

(From the Rudolph Spreckels Physiological Laboratory of the University of California.)

When a hungry[1] Planarian is placed in a finger-bowl of water a piece of liver has been lying for some minutes, the worm moves about the dish until it enters the diffusing meat-juice. It pauses at once and extrudes the pharynx. This waves about for a few seconds and is withdrawn. Then the worm takes up its course directly toward the meat and, reaching it, begins feeding. The same reaction is seen when the worm is gliding on the surface film and a bit of egg-yolk is floated near it. This preliminary extrusion of the pharynx, although regularly appearing when hungry worms first come into contact with meat-juice, is very transitory. It does not occur again if the worm leaves the juice and reënters it on its way to the meat or if it is placed in an extract of meat and left to wander about there.

If, however, a section is made through the worm behind the cephalic lobes and anterior to the pharynx the result differs. Directly after the cut the worms move about in an active manner though they eventually come to rest. If while still moving they enter a region of diffusing meat-juice they often stop. The pharynx is extended and remains so; feeding begins upon the juice itself and may continue until the body has swollen and become lighter colored as in ordinary feeding. Often the reaction is not so prolonged but it is always more free and persistent than in uncut worms. From this a natural inference would be that such pieces placed directly upon meat would respond with the feeding reaction, and this is true. The posterior portion of any hungry worm will feed when placed upon a fresh piece of liver.

[1] Worms which had been fasting several weeks were used for these reactions. Well-nourished worms do not furnish good material, as their sensitivity to chemical stimulation is low compared to that of fasting worms.

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This result is contrary to that of Pearl,[1] and more extended than that of Bardeen.[2]

A certain degree of independent activity on the part of the pharynx is thus indicated. The same thing could be suspected from the feeding reaction of normal worms. Often a normal worm will come to rest side by side but not in contact with a piece of meat. The pharynx will be extruded and directed toward the meat and will stretch out to reach it to such a degree that the portion of the body to which the pharynx is attached will be pulled out of place, making an indentation on the dorsal surface of the worm. The worm itself will not move at all to help the pharynx, whereas if the pharynx were not extended the chemotropic reaction of the whole worm would carry it directly up to the meat.

There seems to be a twofold chemotropic response, that manifested by the movement of the whole worm toward the meat and that manifested by the movement of the pharynx. Both responses are accurately directed, but when the one is active the other is apparently inhibited. Thus when the stimulation is comparatively weak, the sensitive cells of the skin respond in such a manner that the worm moves directly toward the meat and ordinarily the pharyngeal reflex is entirely in abeyance. When, however, the stimulation is very strong, as upon direct contact with meat or upon the first contact of a very hungry worm with meat-juice, the pharyngeal reflex dominates for a longer or shorter time. When the anterior end of the worm is removed, with it are taken away those portions of the skin which are most sensitive to chemical stimulus. Now a weak stimulus, as that of diffusing juice, calls forth a more pronounced pharyngeal response as though some inhibition were removed.

Some independent chemotropic response seemed so certainly to be located in the pharynx that the question of the activity of the isolated pharynx was raised. If a section is made through the anterior extremity of the pharyngeal sac gentle pressure will force out the pharynx which is now entirely separate from the body. It must be observed that pharynxes so prepared vary as much in sensitivity as do the worms from which they

[1] Pearl, R., *Quarterly Journal of Microscopical Science*, 1902–3, Vol. 46, p. 509.
[2] Bardeen, C. R., *American Journal of Physiology* (1901), Vol. 5, p. 1.

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are extracted. But given a sensitive preparation of which any number can be obtained the reaction is as follows: When placed in a dish of water close to a piece of liver, the pharynx elongates actively, its delicate lip is extended into a broad funnel, the walls of which undulate constantly, receiving and being directed by the stimulating substances of the meat-juice. The result is that the funnel is pointed toward the meat, and not only so but the whole pharynx moves forward sometimes several millimeters by a very wormlike series of extensions, contractions and wrigglings until the mouth comes into direct contact with the meat. There is no doubt of the power of the pharynx to orient itself toward the source of diffusing juice and its ability to move toward that source.

The feeding reaction of the pharynx upon reaching the meat is of enough interest to deserve description. Contact of the mouth with the meat causes a wave of peristalsis to pass almost instantly through the pharynx and as a result a mass of food is discharged from the end of the pharynx to which the intestine would normally be attached. Other waves follow in quick succession and the consumed food is discharged at each wave with considerable force. This activity continues for many minutes and results in the accumulation of a surprisingly large heap of food. The shape of the passage through the pharynx is more or less maintained by the masses of food and the little strings of food particles show a tenacity which indicates that the pharynx has added a secretion probably of a mucous nature to the substance passing through it. The experiment can be duplicated with liver extract or yolk of egg dropped carefully on to the bottom of a dish containing water. The pharynx will work its way into the substance and move sometimes a considerable distance in irregular course through it. During the movement it is constantly eating and leaving behind a trail of consumed food. While this reaction is in progress the pharynx bears a remarkable resemblance to a whole organism. The casual observer would immediately call it a living worm. Such entire maintenance of function by an isolated organ is certainly unusual.

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THE ACCESSORY CHROMOSOME IN A FROG POSSESSING MARKED HERMAPHRODITIC TENDENCIES.

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INTRODUCTION.

In the spring of 1916, the writer, while engaged in determining the effects of starvation upon the development of the germ cells and germ glands of larval frogs, noticed an odd chromosomal body in the germ cells of *Rana pipiens*, which simulated the behavior of an accessory chromosome. Owing to the pressure of other work at that time, the matter was neglected until early in the fall of 1916, when a reëxamination of my material led me to believe that the body I had observed the previous spring was probably an accessory or so-called "sex chromosome."

The present paper is a brief preliminary statement of my observations on the peculiar behavior of this body in the germ cells of *Rana pipiens*.

LITERATURE.

So far as the writer is aware, there is little cytological evidence of the presence of an accessory, or sex determining factor in the germ cells of amphibia. Miss King in 1912 described dimorphism in the spermatozoa of *Necturus maculosus* resulting from unequal chromosomal division. This brief paper seems to exhaust the findings upon this particular subject.

There is a great mass of literature dealing with cytological studies of the germ cells of Urodeles, but with the exception of Miss King's paper, inequalities in the distribution of chromatin have never been reported.

King, '07, failed to find any indications of accessory chromosomes in the germ cells of *Bufo*. So far as I am aware, no one seems to have worked with *Rana pipiens*, though the material is excellent in every respect.

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Material and Observation.

In the spring of 1916, April 7, to be exact, the writer collected a large number of *Rana pipiens'* eggs from a string of shallow pools near the University campus. The eggs were allowed to develop in the laboratory. The larvæ resulting were used for various experimental purposes, but many were kept until after metamorphosis. Throughout the spring, numbers of the larvæ were killed at odd times up to the period of metamorphosis, in order to obtain a complete series of stages illustrating the normal course of development of the germ glands. It was in the early maturation stages of the germ cells of the older larvæ, that the body simulating the behavior of an accessory chromosome was first observed.

The germ glands of the larvæ were fixed in Flemming's fluid and also potassium-bichromate-acetic; both fixatives gave excellent results. The sections were cut a thickness of 7.5 μ and stained with iron-alum hæmatoxylin. A counter stain of congo red and orange G was used, but equally good results are obtainable without employing counter stains.

Microscopic examination of the preserved germ glands of the older larvæ revealed the odd fact that the animals were of three kinds: males, females, and larvæ indifferent as regards sex; *i. e.*, hermaphrodites, the germ glands of which contained both male and female cells. Richard Hertwig has fully described sexually indifferent frog larvæ, animals potentially capable of developing into either sex; since his paper first appeared, several other investigators have confirmed Hertwig's findings. In *Rana pipiens* this indifferent condition appears to be fairly common, and is especially marked in some larvæ; the animals retaining their bisexual character, even after metamorphosis, when one or other of the two sexes results.

Further examination of my material showed that in the case of the female larvæ, the maturation processes begin early and usually several weeks before metamorphosis; conversely, in the male, the first maturation changes seldom if ever occur until several weeks after metamorphosis. All stages of the early maturation processes of the germ cells are visible in the glands of female larvæ seven to eight weeks of age.

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The oögonial number of chromosomes in the female larvæ appears to be twenty-six. It was possible to make, however, only a few counts, and this number stands, therefore, subject to revision. The male spermatogonial counts will be discussed later. Two oögonial counts were made from a specimen of *Rana catesbiana*, which also gave twenty-six chromosomes as the diploid number. (See Fig. 1.) The size and shape of the chromosomes in the two species of frog appear very similar.

In the post-synizesis stages of the germ cells of the female larvæ of *Rana pipiens*, the chromatin threads which appear from the dense, tangled, heavily staining contraction figure (Figs. 2 and 3), are thick, beaded structures, which have a marked tendency to arrange themselves into the well-known bouquet figures. At this stage all of the chromatin has become arranged in the form of pachytene threads, except a rather large, heavily staining body usually found adhering closely to the nuclear wall (Figs. 4–6). This body is somewhat irregular in shape, though constant in size; the shape usually assumed is that of a triangle, with a minute, thread-like structure at the apex. In the bouquet figures, this chromatin mass orients itself toward one pole of the cell along with the free ends of the pachytene threads (Fig. 6).

Study of a somewhat similar body in the adult male indicates that the body observed in the germ cells of the female larvæ represents probably the accessory chromosomes. At the stage shown in Figs. 4 and 6, the body appears double in most cells. In other cells, for instance Fig. 5, the body is shown as single. Attempts to trace this structure back through the synizesis stage proved futile in my *Rana pipiens* material, owing to certain stains employed by me in connection with a different problem, but in specimens of *Rana catesbiana* larvæ 70 mm. in length, pre-synizesis stages reveal a structure apparently identical with the post-synizesis X-body of *Rana pipiens*.

In female *Rana pipiens*, soon after the synaptic stages of the maturation process occur, the young oöcytes are formed and further study of the *X*-body is impossible in the larval form. Attention was then turned to the germ glands of young male frogs.

Through the kindness of Dr. B. M. Allen, I obtained a number

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of sections of the testes of young frogs killed several weeks after metamorphosis had occurred. One of these young frogs was a pseudo-hermaphrodite, over a year old. The testes of this animal contained both ripe spermatozoa and large oöcytes. The germ glands, though containing many oöcytes, were true testes; *i. e.*, the animal was a modified male. All stages of the maturation process were visible; in many follicles the oöcytes and ripe spermatozoa occurred side by side.

The sections had been stained with hæm-alum, counter stained with eosin when received by me. This stain serves very well for the maturation divisions, but is not to be recommended for spermatogonial counts, as it renders the outlines of the chromosomes hazy. As a consequence of this, all of my spermatogonial counts were obtained from sections of testes from other animals, stained with iron-alum hæmatoxylin.

Ten or twelve spermatogonial counts gave twenty-five chromosomes as the diploid number. (See Figs. 7–9.) The chromosomes vary greatly in size and shape, from large V-shaped bodies to small straight rods.

In the hermaphrodite specimen a great many prophases of the first spermatocyte division were found. The chromosomes at this stage were undergoing reduction in number, and appeared as thirteen ring and dumb-bell-shaped bodies of varying size. (See Figs. 10–12.) At slightly later stages, the rings assume the dumb-bell shape also, and are connected end to end by fine linin threads. Still later stages show the linin connectives breaking and the chromosomes becoming scattered through the nucleus. There are thirteen chromosomes visible in the cells. Near the end of the early prophase period, twelve of the typical dumb-bell-shaped prophase chromosomes round off somewhat, becoming oval-shaped; one of the dumb-bell bodies, however, retains its shape; this body, because of its peculiar behavior, I have termed the *X*-body. It is not difficult to identify at this stage because of its large size and dumb-bell shape (Fig. 13).

During the early metaphase stages of the first spermatocyte division, the prophase chromosomes, now oval-shaped, except the *X*-body, line up in the equatorial region of the cell to form the metaphase plate. The *X* chromosome takes its position on

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the metaphase plate along with the other chromosomes. The *X*-body is readily detected at this stage by reason of its marked dumb-bell appearance.

Shortly before the chromosomes split, the dumb-bell-shaped *X*-body migrates to one or other pole of the cell, far in advance of the other chromosomes, and comes to lie at the apex of the spindle close to the centrosome (Figs. 14–19). Lateral views of such spindles, with the chromosomes at the metaphase, are difficult to count; the larger chromosomes obscure the smaller in many instances. Usually, however, twelve obviously bivalent chromosomes can be counted at this stage, in the equatorial region of the cell, just previous to splitting of the chromosomes, and one large, dumb-bell-shaped *X*-body, the accessory, at one or the other pole.

The resulting division separates the twelve bivalents into halves, each half migrating toward its respective pole. As a result of this division, an unequal distribution of chromatin to the secondary spermatocytes occurs; one cell receiving twelve chromosomes plus the dumb-bell *X*, the other cell receiving twelve ordinary chromosomes.

A late anaphase of the first spermatocyte division, showing the *X*-body at one pole of the anaphase, the other pole without the accessory, is shown in Fig. 20.

The dumb-bell *X*-body is very conspicuous in many cells in late anaphase stages. It retains the dumb-bell shape, whereas the remainder of the chromosomes are comparatively small, single bodies. In late telophase stages of the first maturation division, all individuality of the chromosomes is lost; the chromosomes clumping together to form a somewhat irregular crescent. In many such stages the accessory chromosome is easily located by the fact that it is so large that half of it projects from the chromatin mass. It may lie horizontal to the long axis of the cell, resting on the other chromosomes, or at times partly imbedded within their mass.

Three first spermatocyte divisions were observed in which the *X*-body appeared as a single and not a dumb-bell-shaped chromosome. Figs. 21–23 show the single nature of the *X* chromosome. In these cells, the *X* is just half the size it appears in other cells.

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At the side of the metaphase plate opposite from the X-body in one of the three cells, another single body, evidently the product of a precocious division, was observed. This is shown in Fig. 23. In this cell, the dumb-bell accessory seems to have divided, the halves passing to opposite poles. This explanation may also hold for the other two cells in which the X-body appears single, though the other half was not observed. There is a possibility that the single appearance of the X may be due to end views of the body, though such a possibility seems slight. The very large size of the X would, in my opinion, preclude any such possibility.

The secondary spermatocytes, resulting from the division of one of these three cells, the cell in which the X-body divided (Fig. 23), would each receive thirteen chromosomes, each cell receiving half of the accessory; whereas, in the case of the remaining two cells, one half the second spermatocytes would receive thirteen chromosomes, the other half, twelve.

Three other cases of dividing first spermatocytes were observed, in which the typical dumb-bell-shaped X-body had migrated very early to one pole of the cell, and at the other pole a large, round chromosome appeared.

The two parts of the X-body in one cell, were unequal in size, one being considerably smaller than the other. (See Figs. 24–26.) The single, round chromosomes at the opposite pole from the X-body in Fig. 24, judging from its size and shape, appears to be the true half of the accessory or X.

The behavior of the chromosomes in these three cells is difficult to explain satisfactorily. It is obvious, however, that the secondary spermatocytes resulting from such divisions would receive unequal amounts of chromatin.

Repeated examination of my material convinces me that the normal distribution of chromatin to the secondary spermatocytes, at the first maturation division, is twelve chromosomes, plus the dumb-bell-shaped X to one cell, and twelve ordinary chromosomes to the other.

In regard to the case of those cells just described in which the X-body appears to have divided precociously, half passing to each pole in advance of the other chromosomes, it is probable

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that we are dealing with abnormal divisions. The same is true, of course, of those cells in which the accessory either divided precociously, half passing to each pole, but one half becoming linked with a smaller chromosome or perhaps a chromatoid body (see Fig. 24), or else the halves of the *X* are unequal.

However this may be, it should not be forgotten that the animal in whose germ cells these anomalies of chromosomal distribution occur, is bisexual; *i. e.*, with the germinal products of both sexes in its glands. There may, or may not be, a connection between such abnormalities of chromatin distribution at the maturation divisions, resulting, presumably, in the production of three kinds of spermatozoa, and the fact that in certain strains of this species of frog, males, females and animals possessing marked hermaphroditic tendencies occur.

It is obvious, however, that the presence of oöcytes in my specimen is not due to any inequality in chromatin sharing of the daughter cells at the maturation divisions, because the oöcytes are formed long before the male maturation period occurs. Such oöcytes may be due to unequal chromosomal division at an earlier period; perhaps in the spermatogonia or even primordial germ cells. In this connection it may be mentioned that in those follicles containing oöcytes, and they are far from uncommon in my specimen, spermatids and mature spermatozoa are also usually found. The two kinds of cells may be found side by side. The fact that the germinal products of both sexes are usually found together in the same follicle suggests that perhaps both oöcytes and spermatozoa may be products of the same cell originally. It might not be amiss to point out here that the oöcytes and spermatozoa found in the same follicles of this animal represent advanced stages in the germ cell cycle, not found in the surrounding follicles. The surrounding follicles contain spermatogonia and prophases of first maturation divisions, stages much younger than spermatozoa and egg formation.

The Second Spermatocytes.

The second spermatocytes resulting from the first maturation division divide at once without an observable period of rest. The telophase of the first spermatocyte division leaves the chro-

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matin matter a solid, somewhat irregular crescentic mass. This mass soon rounds off its angularities and arranges itself along the equatorial region of the cell. The outlines of the individual chromosomes are, for the most part, obliterated at this stage. No further reduction of chromosomes occurs at this division. The dumb-bell-shaped *X*-body is not difficult to identify in the second spermatocyte at the metaphase. Its identification is rendered easy by its large size and peculiar dumb-bell shape. (See Figs. 27–28.) Part of the dumb-bell usually is seen projecting above the metaphase plate. This is well shown in Figs. 27–28.

All attempts to determine the chromosomal number in secondary spermatocytes in my bisexual specimen proved futile, owing to the hæm-alum stain. The dumb-bell *X*-body divides along with the rest of the second spermatocyte chromosomes. No attempt was made to trace the further history of the accessory body in the spermatids and spermatozoa. I hope to take up this matter in another paper.

The Chromatoid Body.

A number of investigators of amphibian spermatogenesis have observed and reported the presence of an oval, homogeneous chromatoid body in the cytoplasm of the germ cells.

King, '07, reported it in *Bufo*, and claims it forms the acrosome of the spermatozoon. Herman has found a similar body in the spermatids of *Salamandra*. Recently Bachhuber reported the presence of such a body in the spermatocytes of the rabbit. It has also been reported for other animals.

In *Rana pipiens* the chromatoid body is readily found in the cytoplasm of the secondary spermatogonia. I have never observed it in the primordial germ cells of very young larvæ. This structure is of fairly large size, oval in shape and stains readily. It bears considerable resemblance to an extruded nucleolus, though probably has an extra-nuclear origin. The position of the body in the cytoplasm varies considerably. In some cells it adheres closely to the nuclear wall; in other cells it is found near the periphery of the cell. During spermatogonial division it does not appear to divide, though it occurs among the

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spindle fibres; actual division of the body was observed but once. (See Fig. 29.) The body is readily distinguished from the chromosomes by its peculiar character, and the position it takes during cell division.

In the primary spermatocytes the chromatoid body is conspicuous in the cytoplasm. Whether it does or does not divide at the first maturation division was not ascertained. In Fig. 24 it is shown passing to one pole, apparently undivided.

I make mention of the behavior of this structure here, in order to disarm, beforehand, the criticism which perchance might arise in connection with this paper, that I may have mistaken the chromatoid body for an accessory chromosome. The origin and fate of this peculiar body in *Rana pipiens* seem worthy of further investigation.

Summary and Conclusion.

1. The oögonial number of chromosomes in *Rana pipiens* and *catesbiana* appears to be twenty-six.

2. The spermatogonial number is twenty-five in *Rana pipiens*.

3. In the synaptene stage of the germ cells of the female *Rana pipiens* larvæ, a chromatin body is found which simulates the behavior of an accessory chromosome.

4. The reduced number of chromosomes in the male of *Rana pipiens* is thirteen.

5. At the first maturation division of the germ cells of a frog possessing marked hermaphroditic tendencies, there is an unequal distribution of chromatin to the daughter cells, the inequality varying in different cells.

6. Perhaps this unequal distribution of chromosomes at the spermatocyte division and the resulting inequality of chromosomal distribution to the spermatozoa may account for the fact that males, females, and animals possessing marked hermaphroditic tendencies are found in this species of frog.

In conclusion, I wish to acknowledge my indebtedness to Dr. B. M. Allen for the loan of several valuable slides and to Dr. W. R. B. Robertson for several excellent suggestions.

April 11, 1917.

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EXPLANATION OF FIGURES ON PLATES I–VI.

All figures were drawn with the aid of a camera lucida. Spencer 2 mm. apochromat oil immersion objective and oc. 18 used. Drawings are accurate with reference to the nuclear material and chromatoid body. The cytoplasm, however, is represented in the conventional way. *ch*, chromatoid body; *C*, centrosome.

Plate I.

Fig. 1. Polar view of metaphase plate in dividing oogonia from ovary of *Rana catesbiana*, twenty-six chromosomes are present.

Figs. 2 and 3. Synizesis stages in young oöcytes of *Rana pipiens* larvæ.

Figs. 4, 5 and 6. Young oöcytes of *Rana pipiens*, showing the pachytene threads and the X-chromosome adhering to the nuclear wall.

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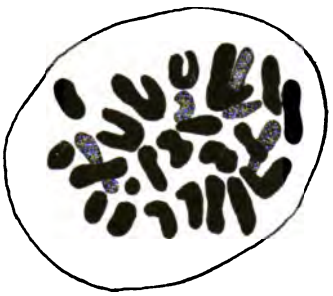

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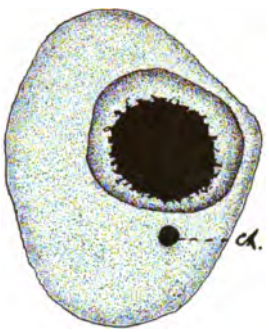

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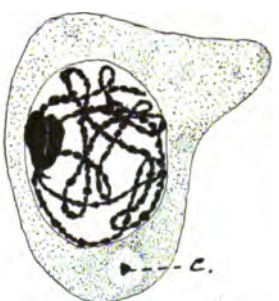

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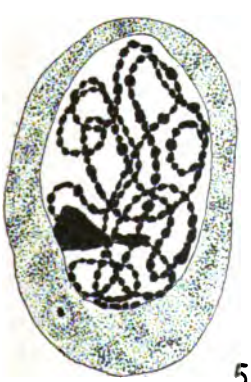

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PLATE II.

FIGS. 7, 8 and 9. Spermatogonia of *Rana pipiens*, showing twenty-five chromosomes.

FIGS. 10, 11, 12 and 13. Spermatocytes, showing prophases of the first maturation division.

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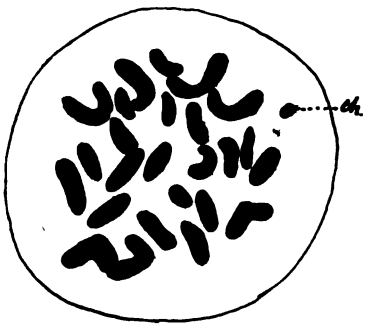

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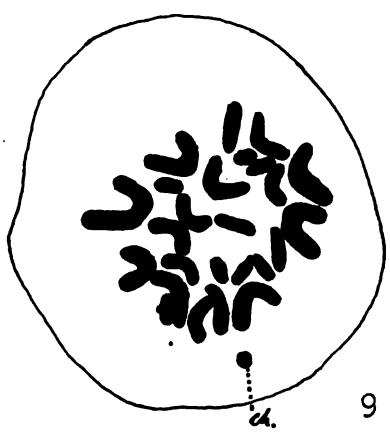

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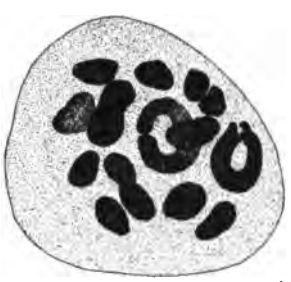

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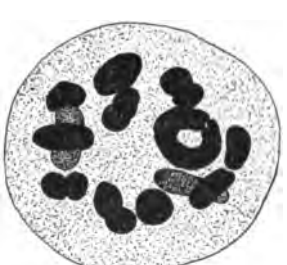

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PLATE III.

FIGS. 14, 15, 16, 17, 18 and 19. Spermatocytes, showing first maturation division and the migration of the accessory chromosomes to one pole.

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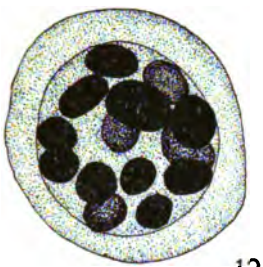

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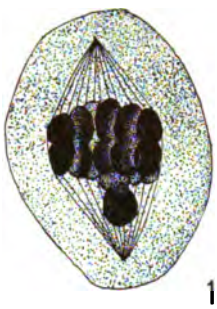

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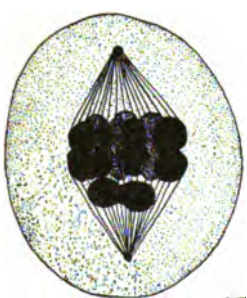

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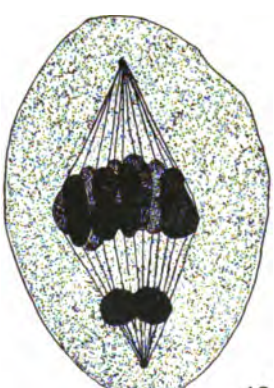

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PLATE IV.

FIG. 20. Late anaphase of a dividing first spermatocyte showing the accessory chromosome at one pole.

FIGS. 21, 22 and 23. First maturation divisions showing single nature of accessory chromosome.

FIG. 24. First maturation division showing accessory chromosome at one pole and single round chromosome at opposite pole.

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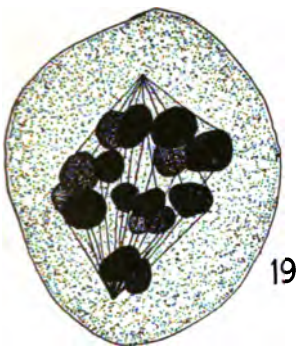

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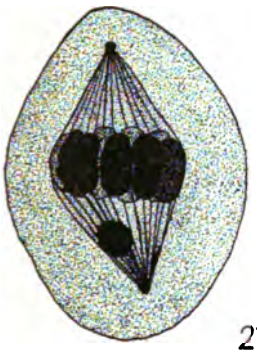

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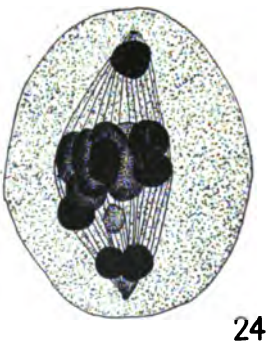

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PLATE V.

FIGS. 25 and 26. First maturation divisions showing accessory chromosome at one pole of the cell; single chromosome at opposite pole.

FIGS. 27 and 28. Second spermatocytes showing the large accessory chromosome.

FIG. 29. Anaphase of dividing spermatogonium showing division of the chromatoid body.

FIG. 30. Spermatocyte of *Rana catesbiana* with two chromatoid bodies in the cytoplasm.

FIG. 31. Spermatogonium of *Rana pipiens* showing large chromatoid body.

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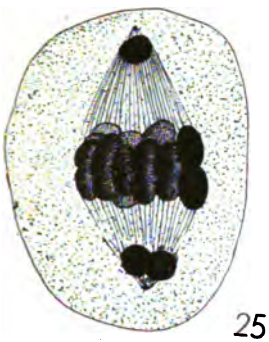
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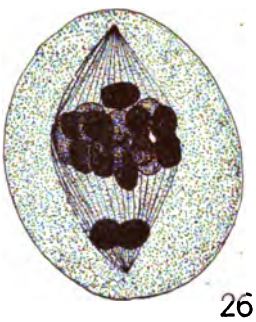
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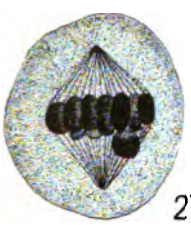
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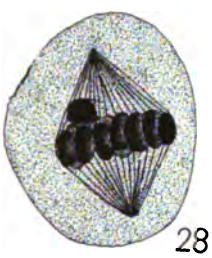
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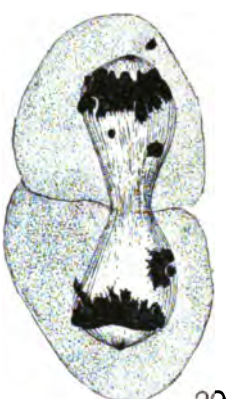
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PLATE VI.

FIG. 32. Oöcyte from the testis of young frog

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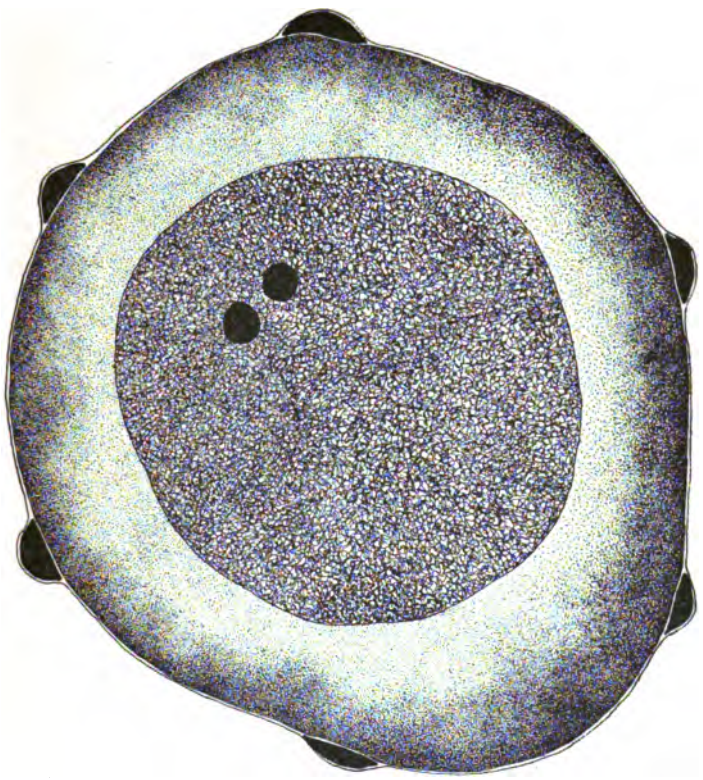

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THE EXPERIMENTAL PRODUCTION OF HYPOTYPICAL OVARIES THROUGH UNDERFEEDING. A CONTRIBUTION TO THE ANALYSIS OF STERILITY.[1]

LEO LOEB.

In former communications we described a condition of the ovaries in which the follicles become atretic, usually before they have reached medium size. Mature follicles are not produced under these conditions and sterility ensues which persists as long as this condition lasts.[1] Such ovaries we designated as hypotypical. We observed those changes especially in guinea pigs in which the corpora lutea had been burnt at a certain period after ovulation. We must assume that the burning of a part of the ovary caused a "tissue shock" in the remaining part of the ovary which, without killing the follicles, weakened the granulosa cells and thus prevented their further development and caused their early disintegration. In addition we found this change in a number of other animals, some of which had not yet been in heat, and had consistently refused copulation, although their age was such that we might have expected to find them sexually active. In some other cases in which as the microscopic examination showed, ovulation had failed to take place after the conclusion of the last sexual period notwithstanding the degeneration of the corpora lutea, hypotypical ovaries were likewise found. This suggests that this condition may be the cause of long-lasting or perhaps perpetual sterility. This hypotypical condition of the ovaries is of theoretical interest, inasmuch as it represents a state of low developmental energy of an organ that is normally in a condition of constant change. From a practical point of view it is of importance because it can lead to sterility of the gonads. It seemed to us therefore of interest

[1] From the Department of Comparative Pathology, Washington University Medical School, St. Louis.

[2] Leo Loeb, *Zentralblatt f. Physiologie*, 1911, XXV., No. 9; *Virchow's Archiv*, 1911, CCVI., 278.

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to carry out further experiments in order to throw additional light on the causes of the hypotypical condition of the ovaries. It occurred to us that a common cause might underlie all those cases in which a local injury of the ovaries was not present, and that lack of proper nourishment might be an important factor in this form of temporary sterility. We therefore carried out four series of experiments in which female guinea pigs received the same kind of food as usual, viz., oats and a little grass or green vegetables, but both in much reduced quantity. We shall discuss our experiments in the inverse order in which they were done. In all cases but one both ovaries were cut in serial sections after the completion of the experiment. In one animal only one ovary was cut serially.

IV. Series.

Five guinea-pigs were used in this experiment. (See Table I.)

Table I.

IV. Series.

Guinea Pig.	Original Weight.	Loss of Weight in Per Cent. of Original Weight.	Period During Which Loss of Weight Occurred.	Result.
A....	294 grams	28%	12 days	Animal weak at end of experiment; about 10–13 days previously ovulation. Hypotypical ovaries of first order.
B.....	309 grams	21% (in 5 days)	6 days	Found dead. Good-sized follicles in granulosa-Degeneration, preparatory to the development of hypotypical ovaries. No ovulation.
B_1....	288 grams	27%	10 days	Weak, killed. Hypotypical ovaries of second order. Delayed ovulation.
C.....	331 grams	36%	14 days	Weak, killed. Hypotypical ovaries of second order. Delayed ovulation.
D.....	342 grams	34%	14 days	Weak, killed. Necroses in liver. Hypotypical ovaries of first order, delayed ovulation.

Microscopic Examination.

Guinea Pig A.—Had lost 28 per cent. of its weight twelve days after beginning of feeding experiment.

In the ovaries: small and small to medium follicles. In the

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latter slow disintegration of granulosa cells takes place and connective tissue grows in. Small follicles in various stages of connective tissue atresia; many follicles in the last stage of atresia, with relative prominence of the theca interna. Remnants of old retrogressing corpora lutea: yellowish stained vacuolar cells surrounded by dense hyaline fibrous tissue, traversed by thick-walled blood vessels. In addition we find well-preserved small corpora lutea with capillaries and myxoid connective tissue in center. Mitoses are not present in the corpora lutea. The *uterus* thin, with low cylindrical surface epithelium and small glands. The mucosa is fibrillar. No sign of a cell-layer.

We must assume that ovulation had taken place at least as early as ten days before examination or even somewhat earlier. The structure of the corpus luteum and the absence of all proliferative changes in the uterine mucosa suggest this conclusion. The new corpora lutea remain small as a result of the insufficient food. The follicles develop only to small-medium size and then undergo atresia. Thus hypotypical ovaries are produced. The uterus is thin, atrophic.

Guinea Pig B.—Examined six days after beginning of feeding experiment; had lost approximately 25 per cent. to 27 per cent. of its weight. This animal was found dead; examination several hours after death.

In the ovaries good-sized follicles with granulosa degeneration; good small and small-medium follicles; various stages of connective tissue atresia. Neither a preserved nor a retrogressing corpus luteum present. The *uterus* is thin with the usual low cylindrical epithelium of surface and small glands. Some epithelial cells at top of papillæ show mucoid transformation; fibrillar mucosa. In this case we have to deal with ovaries which are not yet hypotypical, but which apparently undergo some changes tending towards a hypotypical condition; the larger follicles degenerate and the smaller follicles would in all probability fail to develop to their full size. In this animal ovulation had failed to take place throughout a considerable period of time.

Guinea Pig B_1.—Examined ten days after beginning of feeding experiment; had lost 27 per cent. of its weight. *Ovaries* show

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small and small-medium good follicles. In the latter follicles a slow solution of granulosa sets in. We see some follicles of almost medium size without granulosa, but as yet without connective tissue ingrowth. Follicles in various stages of connective tissue atresia, especially numerous follicles in the last stages of connective tissue atresia with relative prominence of the theca interna. A small yellow fibrous body, the remnant of an atretic corpus luteum is present.

Uterus thin with the usual relatively low epithelium and small glands and fibrillar mucosa. In this case after degeneration of the corpus luteum of the former period, a new ovulation failed to take place and the follicles did not grow to normal size. A hypotypical ovary resulted.

Guinea Pig C.—Examined fourteen days after beginning of feeding experiment; had lost 36 per cent. of its weight. *Ovaries* small, living corpora lutea with small center of fibrous tissue. There is perhaps one mitosis in a Lutein cell. In addition there are fibrous, yellow bodies, the remnants of degenerated corpora lutea. Small and small-medium good follicles. Medium follicles show slow granulosa degeneration. The ingrowth of connective tissue into the follicles is probably somewhat delayed; there are a number of follicles with loss of granulosa and as yet without ingrowth of connective tissue. Various stages of connective tissue atresia of follicles are seen. Atretic follicles in the last stage of atresia and with a large mantle of theca interna are frequent. *Uterus* shows the same character as in the other animals; relatively low epithelium, small glands, fibrillar mucosa. Mitoses nowhere visible.

We may assume that in this case the last ovulation took place very soon after the beginning of the feeding experiment. No trace of proliferation was visible in the uterine mucosa. This indicates that at least ten days had elapsed since the last ovulation, a conclusion in accordance with the character of the corpora lutea. The follicles instead of growing to normal size after ovulation, remained small and underwent premature atresia.

Guinea Pig D.—Examined fourteen days after beginning of feeding experiment; had lost 34 per cent. of its weight. *Ovaries* with small and small-medium good follicles. In the latter slow

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granulosa degeneration sets in, leading to a complete solution of the granulosa cells with subsequent ingrowth of connective tissue resulting in various stages of connective tissue atresia. Follicles in the last stages of connective tissue atresia form a prominent part of the ovaries. Neither a new corpus luteum nor a remnant of a retrogressing corpus luteum visible. The last ovulation had therefore taken place more than four weeks previous to examination. The *uterus* is thin, with relatively low epithelium and small glands. Much secretion is present in surface epithelium and some gland ducts. The mucosa is thin and fibrillar.

In the four guinea pigs in which ten days or more had elapsed since the beginning of the experiment the ovaries had become hypotypical; while in guinea pig B, which was examined six days after the beginning of the experiment, degenerative changes took place in the large follicles, probably preparatory to the ensuing hypotypical changes. In this animal the relative loss in weight had been almost as marked in six days as in the other guinea pigs in a longer period. It seems therefore that a minimum time has to elapse before the hypotypical condition of the ovaries is established. The latter can be obtained in one of the following two ways: First, after the degeneration of all the good-sized follicles following ovulation, the small follicles fail to reach full size. Before they have grown to medium size, the granulosa cells perish and connective tissue grows in. Second, the large follicles degenerate perhaps at a somewhat accelerated rate without an ovulation having preceded this change, and the small follicles fail to reach medium size. The granulosa of the small-medium follicles does not show the karyorrhexis affecting simultaneously a large number of the granulosa cells and characteristic of the beginning atresia of large follicles; isolated granulosa cells may, however, show karyorrhexis.

In all of the animals with exception of guinea pig *D* ovulation had probably taken place within the last month; and in two animals within the last two weeks preceding the time of examination. We are therefore justified in assuming that at the beginning of the experiment the ovaries had been fully developed in these animals in accordance with their initial weight and age. The lack of a sufficient amount of food however prevented

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apparently the corpora lutea from reaching full size. It is furthermore probable that toward the end of the experiment the ingrowth of connective tissue into the cavity of the atretic follicles was somewhat delayed. Still the difference in the behavior of granulosa cells and connective tissue is striking. While the former becomes dissolved as a result of the underfeeding, the latter is still active and organizes the cavity of the follicle. The uterus in all cases was thin, and its mucosa did not show any sign of growth. The ova represent a further element still active under these conditions. In the small-medium follicles in process of atresia mitotic and amitotic divisions of the nuclei take place, as well as a division of the superficial part of the egg into cell-like fragments in which however usually nuclei are not visible.

III. Series. (See Table II.)

Fifteen guinea pigs were used in this series, five, weighing between 534 and 380 grams at the beginning of the experiment, had been bred in Missouri; ten younger ones, varying in weight between 378 and 259 grams, were obtained from Iowa. Three of these animals were well fed during the period of the experiment and served as controls. In addition two other control guinea pigs were examined at the beginning of the experiment. We shall first describe ovaries and uterus of the control animals.

1. *Control Animals.*

Guinea Pig No. 3.—Weight Oct. 18, 474 grams. Nov. 11, twenty-four days after beginning of experiment, weight 613 grams. The animal was found pregnant. The ovaries were normal; they contained good follicles of all sizes, large follicles in granulosa degeneration, good mature follicle as well as mature follicles with ganulosa degeneration. Follicles in various stages of connective tissue atresia. Good corpora lutea as well as degenerated corpora lutea (yellow-fibrous bodies). The *mammary gland* was typically proliferating.

Guinea Pig No. 9.—Weight Oct. 18, 286 grams; weight Nov. 2, 375 grams; examined Nov. 4. *Ovaries:* Good corpora lutea, the cavity of which was filled with connective tissue, retrogressing corpora lutea; good small and medium follicles, various stages

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of connective tissue atresia, especially the later stages are well represented. The *uterus* shows a predeciduomatous cell layer with mitoses in the mucosa. The gland fundi with high cylindrical cells. The *mammary gland* consists of acini with relatively high, well-staining epithelium, but without mitoses, and with rather cellular connective tissue between the acini. We have here to deal with normal sexual organs in an animal at a period about five days after ovulation.

Guinea Pig No. 12.—Weight Oct. 18, 263 grams. Weight Nov. 11, 352 grams. In the left horn of the uterus two pregnancies were found. *Ovaries:* Good corpora lutea; their central cavities are filled with connective tissue; good follicles of all sizes. Follicles with granulosa degeneration; good mature follicles; various stages of connective tissue atresia. *Uterus* is that of pregnancy. In addition to these three guinea pigs, two other guinea pigs obtained from Iowa (*1a* and *1b*) were examined as controls at the beginning of the experiment.

Guinea Pig No. 1a.—Weight Oct. 19, 378 grams. *Ovaries:* Good corpora lutea; the central cavity is almost completely filled by connective tissue, but a small remnant is still left unorganized. Good small and medium and almost large follicles. Some follicles in the last stages of connective tissue atresia. *Uterus* well developed with a predeciduomatous cell layer in mucosa. Fundi of glands with high cylindrical cells. *Mammary gland:* Acini with good cuboidal epithelium and a few mitoses; cellular stroma. We have here to deal with normal sexual organs in an animal about five to six days after ovulation.

Guinea Pig No. 1b.—Weight Oct. 19, 271 grams. *Ovaries:* Good follicles of all sizes, including large and mature follicles. Much hyperemia around the mature follicles, capillaries in theca interna of these follicles much enlarged. Some follicles in various stages of connective tissue atresia, but relatively few follicles in the last stage of connective tissue atresia. No sign of new or degenerating corpora lutea. The *uterus* showed high cylindrical surface epithelium; gland ducts with high epithelium; gland fundi with lower epithelium. The mucosa is hyperemic and succulent and papillomatous. A few mitoses in surface epihelium and at the opening of the glands. *Mammary gland*

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very small. A few acini with good cuboidal-cylindrical epithelium with a few mitoses; stroma between acini cellular. The animal was apparently for the first time in her life in the period of heat. We see then that all the control guinea pigs at the beginning and at the end of the experiment were perfectly normal; they were sexually mature and underwent the typical sexual cycle.

2. *Undernourished Animals.*

In order to shorten the description of our findings, we shall from now on designate ovaries in which atresia sets in when the follicles are as yet of small or small to medium size as hypotypical follicles of the first order, and ovaries in which atresia sets in at a slightly more advanced stage of the development of the ovaries, in follicles which have reached or almost reached medium size as hypotypical ovaries of the second order. The uterus is in those cases thin and shows the characteristics described in the fourth series. We shall designate such a uterus as hypotypical.

Guinea Pig No. 1.—Still active at time of examination. Abortion had taken place recently. Ovaries showed corpora lutea with marked vacuolization and thick-walled vessels. The vacuolization affects all parts of the corpus luteum, but especially the periphery. These are the corpora lutea of the preceding pregnancy; following abortion they underwent degenerative changes. Atretic yellow bodies (the remnants of degenerated corpora lutea); good small and medium follicles and only an exceptional good large follicle. The large majority of the large follicles showed granulosa degeneration; follicles in various stages of connective tissue atresia. One almost mature follicle. *Uterus:* Cells of surface epithelium high and large with prominent cytoplasm; glands with rather high epithelium, but with less prominent cytoplasm; many mitoses in surface epithelium; fibrillar mucosa. At one place there are signs that regeneration had taken place and that a decidua had been present. *Mammary Gland:* Large. Acini with good cuboidal epithelium and small lumen. Some colloid in lumen. Some large vacuoles in epithelial cells. No mitoses. Cellular stroma. Some mononu-

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clears and polynuclears in stroma. Beginning secretion following abortion. In this case the underfeeding was probably responsible for the abortion. In the controls otherwise held under the same conditions, but given sufficient food, pregnancy proceeded normally. Ovaries, uterus and mammary gland corresponded to the stage of the sexual cycle of this animal.

Guinea Pig No. 2.—Weight Oct. 18, 464 grams; Nov. 18, 302 grams. Following this date it received a full ration of food for a few days and on Nov. 25 it had regained a part of its loss, weighing now 357 grams. Underfeeding was again resumed. On Nov. 30, it weighed 303 grams and it was examined. *Ovaries:* Two young corpora lutea with dilated capillaries. There is in the corpora lutea a central cavity surrounded by a little peripheral connective tissue. Mitoses are found in the capillaries and in the central connective tissue. Atretic yellow bodies with vacuoles and thick vessels are also present. Small and small-medium good follicles with a very thin layer of granulosa which even around the egg is imperfect. Some mitoses in the granulosa cells. In other small-medium follicles granulosa is lost. Follicles in various stages of connective tissue atresia. The *uterus* shows a high cylindrical surface epithelium, glands with cuboidal cells and a very cellular myxoid mucosa. The *mammary gland* consists of good acini without mitoses; it presents an intermediate character. Ovulation had taken place about 2–3½ days previous to the examination. It is probable that following ovulation the ovaries ceased to develop normally and are becoming hypotypical. The solution of granulosa cells in small-medium follicles suggests such an interpretation. We may assume that probably following the transitory gain in weight a few days before examination ovulation occurred. For a considerable period previous to the last ovulation, ovulation had failed to take place, probably in consequence of the underfeeding. We find therefore only remnants of degenerated corpora lutea, representing the corpora lutea of the preceding cycle. The underfeeding did not prevent mitotic division in various structures, nor did it prevent the development of a slightly predeciduomatous condition of the uterine mucosa.

Guinea Pig No. 4.—The animal was in a dying condition,

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when examined. Only one *ovary* was examined microscopically. Small and small-medium good follicles; some granulosa degeneration in other small-medium follicles. Various stages of connective tissue atresia; especially many follicles in the last stages of connective tissue atresia, much interstitial gland. No corpus luteum present. *Uterus* showed low cuboidal-cylindrical epithelium of surface and glands without mitoses; fibrillar mucosa. The *mammary gland* consisted of dense fibrous tissue with a few ducts and acini; acini with cuboidal epithelium and without colloid in the lumen. At places the stroma was somewhat more cellular. In this case we can be sure that the ovaries were hypotypical, although only one ovary had been examined. The condition of the uterus makes it certain that a recent ovulation had not taken place. The animal has been markedly affected by the underfeeding, it was in a dying condition at the end of the experiment.

Guinea Pig No. 5.—The animal was found dead. Hypotypical ovaries of the second order. Some medium follicles with granulosa degeneration. Two retrogressing small vacuolar corpora lutea with thick vessels. *Uterus* is hypotypical. In this case ovulation had been delayed.

Guinea Pig No. 6.—Examined in a dying condition. Hypotypical ovaries of the second order. Some almost medium follicles with a thin wall of granulosa. In follicles of almost medium size some granulosa degeneration. No corpus luteum or remnant of a corpus luteum visible. *Uterus* hypotypical.

Guinea Pig No. 7.—Found dead. No noticeable postmortem changes. Hypotypical ovaries of the first order. Small degenerating corpora lutea with vacuolar cells and thick vessels. *Uterus* hypotypical. In this case a new ovulation had not yet taken place, although the corpus luteum of the preceding period was degenerating. The loss of weight in this case had been rapid.

Guinea Pig No. 8.—Found dying. Hypotypical ovaries of the second order. There is present a retrogressing small corpus luteum with vacuolar cells. Uterus hypotypical. Mammary gland shows dense fibrous tissue and small gland ducts without mitoses. Ovulation was delayed.

Guinea Pig No. 10.—Found dead, slight postmortem changes.

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TABLE II.

III. SERIES.

Guinea Pig.	Original Weight.	Loss of Weight in Per Cent. of Original Weight.	Period During Which Loss of Weight Occured.	Result.
No. 1..	534 grams	28%	14 days 30% in 24 days	Abortion. Typical cycle, delayed ovulation.
No. 2..	464 grams	23%	33 days 35% in 43 days	Period of refeeding preceded examination. Ovulation with growth in uterine mucosa a few days previous to examination. Ovulation had been suspended for a long period of time. Beginning hypotypical ovaries.
No. 4..	415 grams	31%	14 days	Dying. Hypotypical ovaries of first order.
No. 5..	380 grams	28%	12 days.	Found dead. Hypotypical ovaries of second order. Delayed ovulation.
No. 6..	313 grams	27%	14 days	Dying. Hypotypical ovaries of second order.
No. 7..	302 grams	In 5 days about 9% of the weight lost; had lost some weight in the preceding 10 days	7 days	Hypotypical ovaries of first order. Delayed ovulation.
No. 8..	305 grams	27%	7 days	Dying. Hypotypical ovaries of second order. Delayed ovulation.
No. 10.	292 grams	In 5 days lost 12% of its weight. Died 2 days later	7 days	Found dead. Hypotypical ovaries of first order. Delayed ovulation.
No. 11.	283 grams	26%	7 days	Dying. Hypotypical ovaries of second order. Delayed ovulation.
No. 13.	259 grams	Lost in 5 days 13% of its weight	7 days	Hypotypical ovaries of second order. Delayed ovulation.
		Controls.		
No. 3..	474 grams	29%	24 days	Well-developed pregnancy. Normal ovaries.
No. 9..	286 grams	31%	15 days	5 days after ovulation. Ovulation evidently occurred at normal intervals.
No. 12.	263 grams	34%	24 days	Pregnancy. Normal ovaries.
		Additional Controls; Examined at Beginning of Experiment.		
No. 1a.	370 grams			About 5–6 days after ovulation. Normal ovaries and uterus.
No. 1b.	271 grams			Animal in first heat. Normal sexual organs.

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Hypotypical ovaries of the first order. A small vacuolar corpus luteum. Hypotypical uterus. In this case again delayed ovulation.

Guinea Pig No. 11.—Was found dying. Hypotypical ovaries of second order. Small vacuolar retrogressing corpora lutea. Uterus hypotypical. Delayed ovulation.

Guinea Pig No. 13.—Found dead. Some postmortem changes. Hypotypical ovaries of second order. Small vacuolar retrogressing corpora lutea. Hypotypical uterus.

In this series all the guinea pigs show hypotypical ovaries after a loss of weight of approximately 26–31 per cent. in the course of one to two weeks. In Guinea Pig No. 13 we find in a period of five days a loss of 13 per cent., but in the last two days before death a further considerable loss of weight had in all probability occurred. In 2 cases (Guinea Pigs Nos. 1 and 2) we do not yet find hypotypical ovaries. In the first animal, however, abortion took place toward the end of the experiment. We may attribute the abortion to the lack of a sufficient quantity of food. Abortion is not usual among guinea pigs without a preceding experimental interference. Following the abortion a new ovulation had not yet taken place at the time of examination. In the second animal (Guinea Pig No. 2) ovulation had taken place shortly before the conclusion of the experiment, but there were indications of a beginning hypotypical condition of the ovaries following the ovulation. In these two cases we have in the first place to deal with somewhat heavier animals, and secondly, a slightly smaller relative loss of weight was distributed over a longer period of time. It is probable that the greater initial weight of these guinea pigs caused a greater resistance to the underfeeding; their general condition was less affected by the underfeeding and consequently we find as yet no marked changes in ovaries and uterus. In the five control animals of this series the sexual cycle took its normal course; pregnancy as well as ovulation proceeded normally.

II. Series. (See Table III.)

Five guinea pigs were used in this experiment. Guinea Pig No. 1 served as control, the other four were undernourished and

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received only water and a few leaves of grass each day. The experiment began June 30, 1916. For briefness sake, only abstracts of the descriptions can be given.

TABLE III.

II. SERIES.

Guinea Pig.	Original Weight.	Gain or Loss in Weight in Per Cent. of Original Weight.	Duration of Experiment.	Result.
No. I, control.	438 grams	A loss of 15 grams	9 days	At end of pregnancy. Normal ovaries and uterus.
No. II.	462 grams	−29%	9 days	Perhaps first change in direction toward development of hypotypical ovaries. Probably abortion a short time previously. Delayed ovulation.
No. III.	454 grams	−38%	9 days	Not hypotypical ovaries, but beginning change tending in that direction. Delayed ovulation.
No. IV.	428 grams	−35% (in 9 days)	10 days	Not yet hypotypical follicles but probably some retardation in the development of follicles. Delayed ovulation. Animal very weak at end of experiment.
No. V..	439 grams	−34% (in 9 days). Then a gain of 24% of the original weight and of 31% of the lowest weight reached July 9	21 days. (9 days deficient and 11 days full feeding)	No mature follicles and delayed ovulation; ovaries otherwise normal.

Guinea Pig No. 1.—Control. In one horn of uterus one embryo: near end of pregnancy. In this case the condition of *ovaries*, *uterus* and *mammary gland* corresponded to the last stage of pregnancy. In the ovaries large and mature follicles were present. The mammary gland was proliferating.

Guinea Pig No. II.—Ovaries: Good small and medium follicles, some larger follicles in granulosa degeneration and perhaps one well preserved large follicle. Most follicles seem to degenerate, when in medium size. Various stages of connective tissue atresia. Large vacuolar corpora lutea with thick vessels. Atretic yellow body (remnant of corpus luteum). *Uterus:* Relatively low cuboidal-cylindrical epithelium of surface and glands. Some

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mitoses in surface epithelium, nowhere else; fibrillar mucosa. *Mammary gland:* Large gland, with acini of various sizes, often filled with colloid material. Fibrous or fibrillar stroma, with small nuclei. Some epithelial cells with vacuoles, indicating slight secretory activity. In this case there is perhaps present a beginning of a change in the direction toward the development of hypotypical ovaries; but as yet the ovaries have not reached this condition. The state of the mammary gland and possibly of the ovaries suggests that an abortion has taken place a relatively short time previous to the examination. The large vacuolar corpora lutea with thick vessels, as well as the condition of the mammary gland, which shows signs of secretion, suggest such a conclusion.

Guinea Pig No. III.—Ovaries: Young corpora lutea. Loose central connective tissue fills the cavity. Atretic yellow vacuolar body with thick vessels (remnant of corpus luteum). Good small, medium and almost large follicles. No large follicles, but a few almost large follicles with granulosa degeneration. A number of small-medium or medium follicles lost their granulosa through a gradual process of solution and form cysts in which the egg is lying free. In other degenerating follicles of this kind connective tissue grows into the cavity. Many follicles in last stage of connective tissue atresia. *Mammary gland:* Good sized, acini with cuboidal epithelium, fibrous stroma. No mitoses. The ovaries are not yet hypotypical, but the condition of the smaller follicles indicates a beginning change in this direction.

Guinea Pig No. IV.—Nine days after the beginning of the experiment the animal was offered a larger quantity of food, but refused to eat it. One day later it was found in a very weak condition and it was examined. *Ovaries:* Corpora lutea vacuolar, with thick vessels; they show beginning degeneration, atretic yellow bodies (the remnants of degenerated corpora lutea). Good small and small-medium follicles. Large follicles show granulosa degeneration. Well preserved large follicles are not present. Follicles in various stages of connective tissue atresia. *Uterus:* Relatively low surface epithelium. Similar epithelium in glands. Fibrillar mucosa without mitoses. *Mammary gland:* small gland in a resting condition with marked development of

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fibrous tissue. In this case the ovaries are not yet hypotypical, but the lack of good medium and large follicles indicates that some inhibition in the growth of follicles occurred, while the large follicles that had developed previously failed to mature and degenerated.

Guinea Pig No. V.—Weight June 30, 439 grams; July 9, 289 grams. From July 10 on a full ratio of food was given daily and the animal gained in weight. On July 21 it weighed 396 grams; it had not yet regained its original weight. On that date it was examined. *Ovaries:* Contained an atretic yellow body (remnant of a corpus luteum). Good follicles of all sizes, including good large follicles. Large follicles in granulosa degeneration and in various stages of connective tissue atresia. *Mammary gland:* Small intermediate, no mitoses. In this case the ovaries are on the whole normal; but apparently mature follicles did not develop and an ovulation had not taken place for a long period of time previous to the examination, despite the fact that the animal had regained part of its weight.

We find then in this series that hypotypical ovaries did not develop as the result of underfeeding; but in Guinea Pig Nos. II., III., and IV. we find some changes which indicate a tendency toward the development of hypotypical ovaries; either a premature atresia sets in in follicles which are only of medium size, or even smaller, or at least a retardation in the development of large follicles apparently occurred toward the end of the experiment. In all cases mature follicles were absent and ovulation was delayed. In the one animal which received a full ratio of food after a preceding period of underfeeding the follicles likewise failed to mature and ovulation failed to take place during the period in which the animal gained in weight. It is, however, possible that the delay in ovulation was not exclusively due to the undernourishment. In one case abortion had in all probability taken place previous to the examination, probably as the result of the underfeeding. In all these cases the relative and especially the absolute loss of weight had been as great as in the guinea pigs of the III. and IV. series, or it was even greater, and yet hypotypical ovaries had not developed. This result may be due to the greater weight of the animals at the beginning of

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the experiment. All the guinea pigs weighed between 400 and 500 grams. These animals were therefore less affected in their general health by the lack of sufficient food than the animals in the other series. Only guinea pig No. IV. showed marked weakness. The duration of the experiment was on the average somewhat shorter than in series III. and IV., and it is possible that this factor also has something to do with the result. But it is probable that the greater weight of the animals in the beginning of the experiment is mainly responsible for the lack of the development of hypotypical ovaries.

First Series of Experiments (See Table IV.).—In this experiment the quantity of food given and changes in weight of the animals had not been determined with the same accuracy as in the later series. While we must consider the possibility of errors in the figures for the weight of the animals it is probable that the error on the whole is not considerable. In most cases the amount of food was increased for several days during the experiment and there was therefore a transitory gain in weight during a certain period. Animals *a*, *b*, *c* and *d* had been thyroidectomized some time previous to the beginning of the experiment; the remaining five animals had been normal.

Guinea Pig No. a.—Ovaries: Atretic yellow bodies (remnants of old corpora lutea). Hypotypical ovaries of second order. Hypotypical uterus. *Mammary gland:* Much gland tissue. Resting, intermediate gland.

Guinea Pig No. b.—March 20, weight 650 grams; April 7, 600 grams; April 30, 280 grams. *Ovaries:* Retrogressing corpora lutea, good follicles of all sizes. Large follicles with granulosa degeneration; follicles in various stages of connective tissue atresia. Not hypotypical ovaries; but no mature follicles developed and ovulation was delayed. *Uterus:* With ordinary surface epithelium and glands. Some vacuoles in surface epithelium. Fibrillar mucosa. Some cystic gland ducts at surface.

Guinea Pig No. c.—March 20, 350 grams; April 7, 275 grams; April 11, 175 grams. Found dead. *Ovaries:* With atretic yellow bodies. Hypotypical ovaries of first order. Hypotypical uterus.

Guinea Pig No. d.—Weight March 20, 500 grams. Receives

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full ratio of food; but loses some weight. April 7, 475 grams; April 10, 400 grams, found dead. Had probably been sick. *Ovaries:* Vacuolar corpus luteum with thick vessels. Small medium and almost large food follicles; some small to medium follicles apparently begin to show connective tissue atresia. *Uterus:* Quiescent with follicular mucosa. *Mammary gland:* Large, resting-intermediate. In this case the loss in weight in the animal caused a condition in the ovaries which is approaching the hypotypical state; there are, however, as yet present some slmost large follicles.

TABLE IV.

I. SERIES.

Guinea Pig.	Original Weight.	Change in Weight in Per Cent. of Original Weight.	Duration of Experiment.	Result.
No. *a*..	700 grams	−46%	24 days	Found dead. Hypotypical ovaries of second order. No maturation of follicles. No ovulation.
No. *b*..	650 grams	−57% (In 23 days −53%)	41 days	Killed. Not hypotypical ovaries. No mature follicles; retrogressing corpora lutea. Delayed ovulation.
No. *c*..	350 grams	−50% (In 4 days 21% loss of weight)	22 days	Found dead. Hypotypical ovaries of first order. No new ovulation.
No. *d*..	500 grams	−20% (In 3 days 15% loss of weight)	16 days	Imperfect control. Found dead. Receives a larger quantity of food; almost large follicles in ovaries but some small-medium follicles undergo connective tissue atresia.
No. *e*..	367 grams	Loses 100 grams (27%) in 1st two weeks, then regains weight	38 days	Control; full ratio of food. About 4½ days after ovulation. Normal cycle took place.
No. *f*..	330 grams	−45%	14 days	Died. Hypotypical ovaries of first order. No new ovulation.
No. *g*..	About 420 grams	−58%	17 days	Died. Hypotypical ovaries of first order. No new ovulation.
No. *h*..	About 420 grams	−58%	21 days	Hypotypical ovaries of second order. No new ovulation.
No. *i*..	About 360 grams	−58%	37 days	Hypotypical ovaries of second order. No new ovulation.

Guinea Pig No. e.—Control. Weight March 24, about 365 grams. April 7, 260 grams; received full ratio. May 1, 359

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grams; examined. *Ovaries:* Two young corpora lutea with capillaries, with mitoses in lutein and endothelial cells. Atretic yellow bodies. Good small and small-medium follicles; follicles in medium and late connective tissue atresia. *Uterus:* Epithelium in glands high; frequent mitoses in gland fundi. In mucosa predecidual cell layer with frequent mitoses. *Mammary gland:* Large ducts, acini with rather high cuboidal epithelium, very cellular, not fibrous stroma; probably mitoses in acini. This is an animal at a period about 4½ days after ovulation. Mature follicles developed and ovulation took place.

Guinea Pig No. f.—Hypotypical ovaries of first order with small, vacuolar, retrogressing corpus luteum with thick vessels. Hypotypical uterus with a few mitoses in gland ducts.

Guinea Pig No. g.—Hypotypical ovaries of first order; corpus luteum with vacuolar cells and dense fibrous nucleus; probably 15–20 days old. Atretic yellow bodies. Hypotypical uterus. Ovulation had taken place approximately at the time of the beginning of the experiment. The atretic yellow bodies indicate that previous to the experiment the sexual cycle had taken its normal course.

Guinea Pig No. h.—Weight March 24, about 420 grams. April 7, weight 220 grams. April 13, 175 grams. Killed April 14. *Ovaries:* Atretic yellow bodies (remnants of corpora lutea); small and small-medium good follicles. In the small-medium follicles atresia sets in and connective tissue grows into the cavity. A few follicles reach almost medium size and then disintegration of the granulosa and connective tissue atresia set in. Only exceptionally a good medium sized follicle present. Hypotypical uterus; some mitoses in surface epithelium. *Mammary gland:* large. Small acini with or without colloid, with low or medium-sized epithelium and partly vacuolar cells. Stroma fibrillar or fibrous. There may have been secretion in this gland previous to the beginning of the experiment.

Guinea Pig No. i.—Hypotypical ovaries of second order with atretic yellow bodies. Hypotypical uterus; some mitoses in surface epithelium. Guinea pigs *h* and *i* were evidently not so strongly affected by lack of food. They did not die spontaneously at the end of the experiment.

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From the results of this series, we may draw the following conclusions: Of the seven animals which were subjected to the low diet, 3 showed hypotypical ovaries of the first order; 3 showed hypotypical ovaries of the second order. In all of these cases ovulation had been prevented. In one case, the ovaries were not hypotypical, although the loss in weight had been just as considerable as in other cases. In this case, however, maturation of follicles and ovulation failed to take place. If we inquire into the condition responsible for these differences in the behavior of different animals, we may suggest that it depends in all probability on differences in the general effect of underfeeding on the animals. Those animals that were alive at the end of the experiment showed a less far-going hypotypical condition than those that had just died at the time of examination. This series shows furthermore that a hypotypical condition may be produced even in a relatively old guinea pig with a considerable initial weight, provided there is a considerable loss of weight extending over a relatively long period of time and a marked influence in the general condition of the animal. If, on the other hand, an animal with a considerable initial weight is less affected generally and its strength is better preserved, throughout the experiment, merely maturation of the follicles and ovulation are prevented from taking place, but a hypotypical condition is not produced. In all those cases in which the ovaries are hypotypical the uterus is thin and shows the resting condition found also in the other series. In this series the loss of weight of the underfed guinea pigs was more considerable than in the other series and the experiment extended over a longer period of time. In the two control animals a hypotypical condition of the ovaries was not found. In one of these the cycle in ovaries and uterus took its normal course; in the other, a loss of weight had occurred, the cause of which remained obscure. While in this case the ovaries do not show a hypotypical condition, some retardation in the development of the follicles had occurred.

Conclusions.

We used in our experiments altogether thirty-four guinea pigs, eight of which served as controls, while twenty-six were sub-

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jected to insufficient feeding extending over periods varying between 33 and 6 days. In the majority of experiments the loss in weight varied between 25 per cent. and 35 per cent. of the initial weight. In some cases the loss was greater, in others less. In the controls there was usually a gain in weight during the experiment, but in two cases there was some loss and in one the loss was as much as 20 per cent. of the initial weight. This animal cannot be considered as a perfect control. In the controls the sexual cycle took its normal course and ovaries and uterus were normal in seven animals. In three of these pregnancy was present which proceeded normally. In several others ovulation occurred during the time of the experiment and the follicles developed in a healthy manner. In the one case, in which the animal lost 20 per cent. of its weight, the ovaries were not yet hypotypical, but some change was noticeable tending in that direction. Of the animals subjected to lack of a sufficient quantity of food the ovaries were hypotypical in eighteen cases. There were differences in the degree in which this condition had developed; while the ovaries of some showed a hypotypical condition of the first order, in others hypotypical ovaries of the second order were present. In the former the development of the follicles proceeded only to that stage in which the follicles were of small-medium size. At this stage atresia set in, while in the second kind a few follicles developed to medium or almost medium size; while others became already atretic at an earlier stage. In no case were large follicles produced in any of these ovaries. In three other cases changes were noticeable in the ovaries which suggested a beginning in the direction toward the development of hypotypical ovaries, while in five animals hypotypical changes were not yet noticeable, but an inhibition of ovulation had occurred owing to the fact that the large follicles failed to mature. We see then that in all the animals a failure of the follicles to mature occurred and in 69 per cent. of the animals the ovaries were hypotypical. In addition pregnancy was found in none of the animals. In one of them abortion had occurred, with certainty and in a second one with great probability, in both cases probably as a result of the underfeeding.

If we now inquire into the causes of the variability in the

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effects of the underfeeding on the ovaries, our experiments suggest very strongly that in addition to the relative loss in weight two other factors may be of significance, viz., first the length of time during which the loss of weight occurs. It seems that more than six days must elapse, before hypotypical ovaries are found. And secondly, the weight of the animal at the beginning of the experiment is of importance. The more the animal approaches full size, the greater is the difficulty with which the changes in the ovaries set in. They are more readily produced in animals weighing between 300 and 400 grams than in animals weighing over 400 grams. But they can be produced in the latter. The effects on the ovaries are therefore to some extent parallel to the effects of undernourishment on the general condition of the animals. Heavier guinea pigs who suffer the same percentage in loss of weight as smaller animals are apparently very much less affected by it as far as their general health is concerned. It is probable that the greater the portion of the food intake is which the animals have to set apart for growth, the more the portion to be used for maintenance is diminished and it seems furthermore that the effect on the general health and on the condition of the ovaries depends upon the portion available for maintenance. The state of the ovaries determines the condition of the uterus, which is always in a resting, almost atrophic condition in cases in which the ovaries are hypotypical. In a similar manner the condition of the mammary gland depends upon the state of the ovaries. We see then that as a result of underfeeding there takes place an increased destruction of granulosa cells which affects even follicles of small or small-medium size. This degeneration of the granulosa cells at such an early stage of the development of the follicles does not occur in the form of a massive destruction, but here and there cells in the rows of the granulosa adjoining the cavity of the follicles are dissolved. While we may notice cells in which the nuclei undergo karyorrhexis, it is probable that other cells perish without a distinct karyorrhexis taking place. If, on the other hand, a degeneration sets in in the larger follicles in normal ovaries, a large number of granulosa cells are destroyed simultaneously and the nuclei of all these cells show karyorrhexis.

We must therefore assume that if the amount of available food

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stuffs is diminished below a certain quantity, those cells perish first which are farthest removed from the source of nourishment which is furnished by the capillaries of the theca interna. But the destruction is not limited to the cells adjoining the cavity, but affects in the end the whole granulosa. We see then that under the condition of lack of proper nourishment, a condition which otherwise would occur much later, is observed at an early period of the development of the follicles, and the process of destruction in this case takes place more slowly than in the case of atresia of the large follicles. It is in the small follicles a more chronic, in the large follicles a sudden, acute process. Those granulosa cells in the small and small-medium follicles which survive for a certain time show approximately the same proliferative energy as the granulosa cells of similar follicles in normal ovaries as the investigations of L. S. N. Walsh have shown.[1] We must therefore assume that the remaining cells are fairly healthy and respond to the stimulus to multiply in a way similar to the granulosa cells in normal follicles, while those cells that are markedly affected by the lack of nourishment become dissolved. We have here to deal with a phenomenon similar to the one which we observed in the case of stationary or retrogressing tumors. In our experimental analysis of tumor growth we noticed that in tumors that had ceased to grow mitoses could still be found rather frequently during a certain period following cessation of growth. This observation led us to the conclusion that the stationary or retrogressing condition was brought about not so much through a complete cessation of growth, as through an increased destruction or solution of cells.[2] We see that in the hypotypical follicles the smallest follicles resist, while in the follicles in which a cavity begins to form processes of degeneration set in. We observe the same phenomenon in the destruction of follicles which takes place at the time of ovulation. Here also the largest follicles perish first and the smallest follicles are most resistant. We must assume either that with the growth of the follicles the granulosa cells undergo changes in their constitution, which makes them more sensitive to injurious

[1] Walsh, L. S. N., *Journal Exp. Medicine*, 1917.

[2] Loeb, Leo, *Virchow's Archiv*, 1902, CLXVII., p. 175; 1903, CLXXII., 345; also E. P. Carson White and Leo Loeb, *Centralbl. f. Bact.*, 1910, LVI., 488.

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influences, or that with the growth of the follicles the nourishment becomes more difficult, and that this unfavorable state of the cells makes them more vulnerable, if injurious conditions arise. In case the second interpretation should be correct we would have to assume that the process of degeneration leads auto-katalytically to further destruction of the follicles of the remaining granulosa cells.

These observations lead furthermore to the conclusion that the formative stimuli, which call forth cell proliferation, are not identical with the food stuffs on which ultimately the life of the cells depends and which are therefore necessary for cell multiplication, a conclusion in full accord with other facts which may be discussed in another connection. Our previous observations and especially the additional observations of Walsh enable us to state the character of the formative stimulus which acts on the granulosa cells. As far as we can determine this stimulus emanates from the egg, and affects principally those granulosa cells nearest the egg, but to a less extent also the more distant cells. It is probable that this stimulus increases simultaneously with the growth of the follicles from small to medium size. In the medium-sized follicles it reaches a maximum. From now on certain unfavorable factors begin to make themselves felt with increasing severity and they lead ultimately either to a degeneration of the granulosa or to that increased differentiation which is characteristic of maturation of the follicles. Perhaps it is the difficulty in the nourishment of the granulosa—a difficulty which increases with the further enlargement of the medium follicles—which is the factor which counteracts the cumulative action of the ovum and prevents a steady increase in proliferative power of the granulosa cells, and which ultimately leads to their death. Thus may be explained the typical growth curve consisting of an ascending and a descending branch as has been described by Walsh. As we stated above, the ova in the follicles which in the hypotypical ovary undergo a premature atresia, show progressive changes. Thus the same factor that causes destruction of the granulosa cells acts on the eggs as a stimulus. In a similar manner connective tissue cells show activity, migrate into the follicular cavity and thus contribute to the atresia of follicles under conditions in which the granulosa cells perish in

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consequence of the lack of proper nourishment. We may then conclude that the connective tissue is more resistant to withdrawal of food than the granulosa cells; and this is a conclusion which is borne out by the behavior of the connective tissue under other conditions. This relatively great resistance to lack of food is one of the most important characteristics of the connective tissue which leads to interesting consequences under normal and pathological conditions. While on the whole the connective tissue cells are more resistant than the granulosa cells yet we noticed that under the conditions of ill nourishment prevailing under our experimental conditions, the ingrowth of connective tissue into the follicular cavities was not rarely somewhat retarded. In such cases we see the follicle forming a cyst lined by theca interna in which the ovum lies free or almost free and in which connective tissue has not yet grown.

The atresia of follicles is comparable to a condition which in other organs is designated as cirrhosis. In the case of the atresia of follicles this cirrhotic process is a substitutive one; the stimulus which induces the connective tissue to become active consists in the loss of the granulosa cells which formerly covered the theca interna. As we pointed out on former occasions, we have reason to assume that there exist in addition stimuli of a different character which call forth cirrhotic processes.[1]

From our investigations it follows that in the guinea pig underfeeding prevents maturation of the follicles and thus causes sterility which lasts as long as the effect of the underfeeding is present in the ovary. This lack of maturation we found in all our cases. In addition we found in the large majority of those animals which had become more markedly affected by the lack of sufficient food, especially in those which had not yet reached their full size, a much farther-going deficiency in the development of the follicles, a condition of the ovaries to which we applied the name "hypotypical." The latter state is altogether incompatible with fertility. The problem that is as yet unsolved concerns the length of time during which this condition is present, and whether the ovaries resume their normal life very soon after the quantity of food has again been increased, or whether the hypotypical condition and lack of maturation

[1] Loeb, Leo, *Journ. Amer. Med. Ass'n*, 1915, LXIV., 726.

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persists at least for some time after the resumption of feeding. Further investigations will have to solve this problem. Our results may perhaps also have some bearing on the condition of the ovaries during pregnancy. We have shown that in guinea pigs ovulation does not occur during pregnancy unless the corpora lutea have been extirpated. In other species however, and especially in man it seems that during pregnancy a maturation of the follicles is lacking. Our experiments suggest that this may depend upon a relative insufficiency of food available for the ovaries during pregnancy; thus an approach to a hypotypical condition of the ovaries would be produced.

Summary.

Underfeeding, if very pronounced, prevents maturation of the follicles in the ovaries of the guinea pigs in all cases and in the large majority of cases leads to the production of hypotypical ovaries in which atresia of follicles sets in before the follicles have reached medium size. Underfeeding leads to a premature solution of granulosa cells. Connective tissue is more resistant to lack of food than the granulosa. The *uterus* in cases of underfeeding is in a resting or atrophic condition. Thus underfeeding produces at least temporary sterility. Those cells farthest removed from the blood vessels suffer first as the result of underfeeding, die and become dissolved, while in those granulosa cells which remain alive the growth stimulus which in part at least emanates from the ovum causes for a short time a normal cell proliferation. Just as the underfeeding produces more pronounced general effects in younger animals, the effect on the ovaries is likewise more marked in younger animals. Through underfeeding it is, however, possible to produce a hypotypical condition even in old guinea pigs. In those animals which had been pregnant at the beginning of the experiment underfeeding led to abortion.

There exists a noteworthy analogy in the relation of cell proliferation to cell destruction in the hypotypical ovaries and in stationary or retrogressing tumors.

Our results emphasize the distinction between food stuffs and proliferative cell stimuli and they may in addition throw light on the condition of the ovaries during pregnancy.

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EXPERIMENTS WITH FEEDING THYMUS GLANDS TO FROG LARVÆ.

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INTRODUCTION.

In 1912, J. F. Gudernatsch published an account of his investigation concerning the effects of feeding various glands of internal secretion to frog larvæ. Among other interesting results observed, was the significant one, that fresh thymus gland, when fed to tadpoles, stimulates the growth processes of these animals and at the same time inhibits the onset of metamorphosis.

In a later communication, January, 1914, this author repeated his previous experiments with thymus feeding, and confirmed his earlier results. For an understanding of the relation between growth and the secretory products of the thymus gland, the importance of the results obtained by this investigator warranted a repetition of his work, which, so far as the writer is aware, has never been questioned. It was with this end in view that the following experiment was undertaken.

LITERATURE.

The experimental researches upon the thymus gland may roughly be divided into two groups: those dealing with the relation between the thymus and bodily growth, and those concerned with the correlation in function of the thymus and sex glands. The literature dealing with the former problem is more extensive than that concerned with the latter.

EFFECTS OF EXTIRPATION UPON GROWTH.

One of the first investigators of this problem was Carbone; this experimenter extirpated the thymus of rabbits, and one dog, and found that the animals did not differ in their development from control animals of the same litter.

Another investigator, Ghika, removed the thymus from eight

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cats and thirteen rabbits; he found that in the first few days there was emaciation and arrest of development which lasted for weeks, although, in some of the animals, the losses were afterwards made good.

Cozzolino removed the thymus from young rabbits weighing 300–400 gm., and in the first two months after the operation was unable to detect any change in their condition.

In a later communication, however, he described crookedness of the legs, arrest of growth, and progressive cachexia in one animal upon which he had operated three months previously.

S. Vicent extirpated the thymus of guinea pigs and did not observe any changes in their condition.

Paton and Goodall found that extirpation of the thymus produced no effect upon the general growth and development of new born guinea pigs.

R. Fischel extirpated the thymus of goats, rabbits and dogs, and obtained negative results. He concluded from his experiment, that thymectomy has no effect upon the development of the subject.

The work of this author has been severely criticized by Basch, who claims that the experimental conditions were inappropriate.

Basch extirpated the thymus of suckling dogs and obtained, striking changes in the growth of his thymectomized animals. The most important growth changes were those affecting the skeleton.

U. Soli found that thymectomy was followed in young rabbits by changes in the development of the skeleton, these changes affecting the hollow, long bones and ribs. This author failed to confirm the results obtained by Basch, with guinea pigs, in which thymectomy is a comparatively simple operation. This would seem to indicate that the symptom complex, observed by Basch following thymectomy in his animals, was probably due to post-operative changes and not connected with the thymus, because in those animals used by Basch, thymectomy is a very severe operation.

Halnan and Marshall extirpated the thymus of young guinea pigs, and found that removal had no influence upon the growth of these animals.

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Pappenheimer extirpated the thymus of young albino rats and found that removal had no effect upon body growth and development.

Adler extirpated the thymus of tadpoles and found that these animals suffered no ill effects from the operation. Growth and development was unaffected.

(The work of Gudernatsch has been noted.)

This rather extensive review of the literature was thought justifiable in view of the discrepancy in the results obtained by the various investigators.

Material and Observations.

The following experiment began May 3, 1915, and was completed July 29, 1916. The work in 1915 dealt with the effect of feeding thymus powder to tadpoles, the work for 1916 was concerned with those experiments in which only the fresh gland was employed as food. I shall discuss the work dealing with the fresh gland first, and devote the latter part of this paper to feeding experiments with thymus powder.

Feeding Fresh Thymus Gland.

This work began April 12, 1916. April 2, the eggs of *Rana pipiens* were gathered from a group of shallow pools near the University campus at Lawrence, Kansas, and allowed to develop in the laboratory. One group of eggs was hastened considerably in development by keeping them in a warm room. When the young larvæ had emerged from their gelatinous egg capsules and developed to the free feeding stage, they were placed in glass bowls ten inches in circumference by four inches deep. The tadpoles were covered with ordinary tap water which was changed daily.

When the larvæ had attained a length of nearly 13 mm., one hundred and fifty were selected, and divided into two groups of seventy-five each; one lot was destined for thymus feeding, the other for control purposes. These two lots of seventy-five animals were then subdivided into lots of ten and placed in glass bowls, one lot to a bowl. Both control and experimental larvæ were reared under identical conditions as regards temperature, light and water supply.

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A second series of larvæ, one hundred in number and averaging 10 mm. in length, were selected and treated the same as the series above. Fifty larvæ were for thymus feeding and fifty for control purposes. These larvæ were from a batch of eggs gathered a few days later than the larvæ of the first series. Hence, throughout the following experiment, the series averaging nearly 13 mm. in length when started upon the thymus feeding I shall designate as Thymus No. 1, the series averaging 10 mm., at the beginning of the work, as Thymus No. 2.

TABLE I.

APRIL 12.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
12	11	10.5	10
12.5	13	11	9
11.5	12	9	11
13	11	10	9.5
13	12	10.5	10.5
13.5	13	10	10
12.5	13.5	9	9
11	13	11	9.5
12	12.5	9.5	9
12.5	11.5	10.5	10.5
11	14	10	11
11	13	11	11.5
13	13	10	10.5
13.5	14	10.5	11.5
12.5	13.5	9.5	11
11.5	11.5	9	11.5
11	12.5	11	10
12	13	9.5	10.5
13	13.5	10	9.5
13	13	10.5	10
Av. len.....12.25	12.6+	10.1	10.25

The experimental larvæ of both series were each day fed finely shredded bits of the fresh thymus glands of calves 3–12 months of age. Only the glands of young animals were used. The glands were obtained from a nearby slaughter-house, and extirpated by myself.

The control animals were fed fresh beef liver each day. Care was taken to give as nearly as possible the same amount of protein material to the animals in each container and of each

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group. Both thymus- and beef-fed larvæ ate greedily the food given them.

At the beginning of the experiment twenty larvæ of both the control and thymus-fed groups of larvæ were measured with a millimeter rule, and the average length computed. This seemed a satisfactory method of procedure, considering that all of the larvæ came from the same batch of eggs, and consequently were of the same age.

A very convenient method of measuring the young larvæ is to lay them upon moistened blotting paper. The moisture in the paper prevents injury to the tail and at the same time the larvæ are unable to lash about.

Table I represents the measurements of twenty larvæ of both control and thymus-fed groups April 12, the day the experiment began.

None of the larvæ measured revealed any indications of limb development when examined under the microscope.

Observations of April 20.

When examined upon this date no observable differences in color or activity of the control and thymus-fed larvæ were noticed. None of the larvæ possessed limb buds. Considerable growth, however, had occurred in the larvæ of both groups, but in regard to length and body size, both control and thymus-fed animals were about equal, as Table II indicates.

Observations of April 29.

A careful examination upon this date, of the larvæ of both thymus-fed and control groups failed to reveal any differences in pigmentation, activity, or mortality among the animals. Upon examination under the microscope, tiny limb buds of the posterior extremities were observed on those larvæ of the control group measuring 25 mm. or over. Limb buds were observed on seven of the thymus-fed larvæ measuring 27 mm. and over, in the thymus No. 1 group.

The limb buds of the larvæ at this early stage appeared as two round, blunt projections somewhat anterior and lateral to the anal opening.

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TABLE II.

APRIL 20.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
18	18	14	15
19	18.5	15	15.5
18.5	19	14.5	15.5
18.5	19	14	14.5
18	19.5	15.5	16
20.5	19.5	16	14
19.5	18.5	16	14.5
20	17.5	15	15
18.5	18.5	15.5	14.5
18	17	14.5	16
19	18	16	16.5
19.5	19.5	15	15
18	19.5	15	15.5
18	18.5	14.5	16
17.5	18	14.5	15
18.5	20	14	15.5
17.5	17.5	16	15.5
19	18	15.5	16
19.5	18.5	15	14.5
18	20	15.5	15.5
Av. len.....18.65	18.62+	15.05	15.22+

The length of the larvæ in both control and thymus-fed animals was about the same though several slight discrepancies are revealed in Table III. The thymus No. 1 group shows a slight acceleration in growth (average length) when compared with their liver-fed controls. This acceleration was not shown by the thymus No. 2 group. These small growth differences are shown in Table III.

The slight difference in the length of the thymus-fed and control animals may possibly be due to the fact that thymus tissue is somewhat more compact than liver tissue and probably presented greater difficulty for the tadpole in eating. Both kinds of tissue were, however, greedily devoured by the larvæ.

OBSERVATIONS OF MAY 4.

Very faint differences in pigmentation were noticed between the larvæ of the thymus-fed and control groups. The thymus-fed animals were slightly darker than their controls which had a reddish tinge. As regards activity, body-shape and mortality,

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TABLE III.

APRIL 29.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
26 l.b.	25.5 l.b.	23	22
25 "	26 "	22.5	23.5
24.5	25 "	21.5	23
28 "	25.5 "	22.5	22.5
28 "	25 "	22	22
25	25.5 "	23.5	21.5
29 "	30 "	22	21.5
25	25.5 "	21	22.5
26.5	24.5 "	21.5	22.5
27 "	29 "	23	22
27 "	23.5	23.5	23.5
25	24	22.5	23
25	23	22	23.5
24.5	24.5	22	22.5
24.5	23.5	23.5	22
26 "	25 "	23	23.5
25	23.5	22	22
27	24	23.5	22
26	24	22.5	23.5
25.5	23	22	22.5
Av. len....25.97	24.97[1]	22.45	22.55

no differences between the animals of the two groups were observed. When examined under the microscope, both the control and thymus-fed larvæ had hind limb buds. The limbs had not as yet differentiated into their two primary divisions, nor had the toe points developed. The limb buds of both groups of animals appeared to be of nearly the same size and in the same stage of development. The thymus No. 2 group of larvæ had by this time outstripped the thymus No. 1 group in growth.

The table below shows that the control animals of the thymus No. 1 group averaged, upon this date, somewhat greater length than any of the thymus-fed larvæ. The animals of the thymus No. 2 group were somewhat larger than their controls.

OBSERVATIONS OF MAY 8.

The thymus-fed larvæ upon this date were distinctly darker in color than their controls; no other differences were observed in the two groups of tadpoles. Both controls and thymus-fed

[1] l.b. = limb buds.

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TABLE IV.

MAY 4.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
28 l.b.	29 l.b.	31 l.b.	26 l.b.
30 "	31 "	33 "	33.5 "
29 "	37.5 "	34.5 "	34 "
28.5 "	34.5 "	32.5 "	25.5 "
27.5 "	33 "	33 "	32.5 "
32 "	30.5 "	27.5 "	32 "
34 "	28 "	30.5 "	30 "
28.5 "	37 "	30 "	26.5 "
34.5 "	30 "	35 "	33 "
32 "	33.5 "	32 "	29 "
28.5 "	30.5 "	33.5 "	25.5 "
29.5 "	37 "	37 "	33 "
34 "	33.5 "	31.5 "	30 "
30 "	34.5 "	30.5 "	29 "
26.5 "	32.5 "	30 "	25.5 "
33 "	34.5 "	31.5 "	29 "
31 "	34 "	32 "	27.5 "
27.5 "	32 "	32.5 "	30 "
30 "	30.5 "	34 "	31 "
34 "	33 "	33 "	32 "
Av. len....30.42	32.8	32.22	29.72

larvæ possessed hind limb buds; these were plainly visible without the aid of a hand lens. The larvæ of both groups of animals had increased in size considerably but the average length of the larvæ of each group varied but little, and such variations are negligible. This is clearly indicated in Table V.

OBSERVATIONS OF MAY 13.

When examined the larvæ of both control and thymus-fed groups had increased in length considerably. All of the tadpoles of both groups had developed hind limb buds and most of the limb buds of the thymus-fed larvæ had differentiated into their two primary divisions and had developed the toe points. The controls seemed somewhat behind the thymus-fed larvæ in respect to the length of the limbs. The pigmentation of the experimental animals was darker than the liver-fed controls.

In other respects the larvæ of the two groups appeared the same. Table VI indicates that the larvæ of all groups averaged nearly the same length. The differences are negligible.

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Table V.

May 8.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
31.5 l.b.	31.5 l.b.	31.5 l.b.	35 l.b.
30.5 "	37 "	33 "	31.5 "
31 "	34 "	34 "	32 "
30.5 "	37 "	32.5 "	33.5 "
30 "	34 "	34 "	30.5 "
36.5 "	33 "	35 "	30 "
40.5 "	37.5 "	34 "	35.5 "
29 "	38 "	33 "	34 "
30 "	33 "	33.5 "	32.5 "
33.5 "	30.5 "	32 "	30 "
33 "	37 "	33 "	32 "
32 "	33 "	37 "	33 "
30.5 "	34.5 "	34 "	31.5 "
33 "	32 "	33 "	30.5 "
29.5 "	33 "	30.5 "	29.5 "
29 "	31.5 "	35.5 "	31.5 "
30.5 "	33 "	32 "	32 "
33.5 "	34 "	31.5 "	32.5 "
32.5 "	32 "	33 "	33 "
34 "	31.5 "	30.5 "	33 "
Av. len....32.02	33.85	33.12	32.15

Table VI.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
37.5 l.b. p.d.[1]	37 l.b.	40 l.b.	44 l.b. p.d.
44 " p.d.	36.5 "	45 " p.d.	45 " p.d.
37 "	42 " p.d.	37 "	39.5 "
33.5 "	33.5 "	37.5 " p.d.	40 "
36.5 "	40 " p.d.	35 "	33.5 "
33 "	35.5 "	33.5 "	45 " p.d.
35.5 "	35 "	33.5 "	40 " p.d.
32 "	37 "	32 "	35.5 "
35 "	38 " p.d.	35.5 "	43 " p.d.
32.5 "	37 "	40 " p.d.	38 "
35.5 "	40 " p.d.	40 "	35.5 "
42 " p.d.	48 "	41.5 " p.d.	39 "
40 "	35.5 "	37 "	38 "
42 " p.d.	33.5 "	38 "	42 " p.d.
43 " p.d.	35 "	34.5 "	42.5 " p.d.
36.5 "	34 "	37.5 " p.d.	41 " p.d.
37 " p.d.	35.5 "	35 "	35.5 "
35.5 "	37 "	34.5 "	34.5 "
34 "	38 " p.d.	37 " p.d.	39 "
39 " p.d.	36.5 "	35 "	37 "
Av. len. 37.05	37.22 +	36.95	39.37 +

[1] The letters p.d. indicate that the limb buds have differentiated into their two primary divisions.

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Observations of May 20.

Examination of the larvæ at this date revealed no difference between the animals of the control and thymus-fed groups as regards pigmentation, body shape, size or activity. The limb buds of both groups had by this time differentiated into their two primary divisions, and had well developed toe points. The size of the limbs of both groups appeared to be the same. The dark pigmentation of the thymus-fed larvæ had gradually faded out, and was no longer visible on May 20. Table VII shows the average lengths of the animals of the experimental and control groups. The average length is nearly the same in all groups. The greatest difference in length between the thymus-fed and control larvæ was about two millimeters.

Table VII.

May 20.

Length.			
Thymus No. 1, Mm.	Control of Thymus No. 1, Mm.	Thymus No. 2, Mm.	Control of Thymus No. 2, Mm.
46 l.b. p.d.	45 l.b. p.d.	44 l.b. p.d.	41 l.b. p.d.
45 " "	45 " "	47 " "	42.5 " "
38 " "	47 " "	39 "	44 " "
46 " "	45 " "	43 " "	39 "
37.5 " "	35.5 " "	41.5 " "	38.5 "
39 " "	37 " "	40.5 " "	41.5 " "
48.5 " "	34.5 " "	45.5 " "	39 " "
37 " "	44 " "	42 " "	40.5 " "
41 " "	46 " "	43 " "	40.5 " "
39 " "	37.5 " "	47 " "	41 " "
39 "	44 " "	46.5 " "	39 " "
42 "	37.5 " "	44.5 " "	41.5 " "
40 " "	46 " "	38.5 "	43 " "
44 "	35.5 " "	40.5 " "	44 " "
37.5 " "	46 " "	39 "	40.5 " "
39 " "	44 " "	38 "	41 " "
38 " "	43 " "	44 " "	39.5 "
39.5 " "	37.5 " "	42 " "	41.5 " "
40.5 " "	38 " "	41 "	38.5 "
41 " "	39.5 " "	39 "	40 " "
Av. len. 40.8	41.37	42.27	40.8

All larvæ which had developed the two primary divisions of the legs in this series, had well-developed toe points.

The larvæ were not measured again until June 8, eighteen days from the date of previous measurement. The larvæ had

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grown so large that it was considered best to avoid handling them, as much as possible. During the eighteen-day interval only four tadpoles died. One was a control and the other three were thymus-fed animals. The four larvæ became bubbly, *i. e.*, air vacuoles appeared in the region of the lungs under the skin. The larvæ came to the surface and floated on their backs; death usually resulted within a few days. The following table indicates the measurements of the larvæ.

TABLE VIII.

OBSERVATIONS JUNE 8.

Measurements of the Larvæ.											
Thymus No. 1, Mm.			Control of Thymus No. 1, Mm.			Thymus No. 2, Mm.			Control of Thymus No. 2, Mm.		
50	Legs	3.5	54.5	Legs	6.5	47.5	Legs	3	50	Legs	2.5
47	"	3	56	"	10	57	"	4	50	"	2.5
57	"	8	50.5	"	9	55	"	4	50.5	"	2
49	"	2	60	"	10	48	"	3	55.5	"	3
60	"	8	45.5	"	2	44.5	"	2	55	"	4.5
52	"	3	43.5	"	8.5	60	"	10	54.5	"	6.5
48	"	2.5	58	"	8	55	"	4.5	51.5	"	2
55	"	2	58	"	10.5	42.5	"	2	53	"	3.5
45	"	2.5	57	"	6	50	"	3	47.5	"	2
58	"	2	60	"	7	53	"	3.5	55	"	4
49	"	2.5	56	"	5.5	41	"	2	45.5	"	3
60	"	3	48	"	3	58	"	7.5	53.5	"	5.5
51	"	4	52	"	4	50.5	"	5	53	"	5
53	"	3	54	"	4	58	"	5	58.5	"	9
58	"	6.5	45	"	2.5	50	"	2	56	"	6.5
57	"	9	59	"	3.5	55	"	3.5	52	"	4
55	"	6	48	"	2	55	"	4	45.5	"	2.5
50	"	5	49.5	"	2.5	60	"	8	49	"	2.5
56	"	5.5	51.5	"	3	50	"	2	51.5	"	3
60	"	6	53	"	4	62	"	11	53	"	5.5
Av. len. 53.95		4.35	52.95		5.57+	52.6		4.45	52.		3.95

OBSERVATIONS OF JUNE 8.

The larvæ of both control and thymus-fed cultures had grown considerably in the eighteen-day interval between the observations of May 20 and June 8. All of the tadpoles of both groups possessed well-developed limbs, some measuring 11 mm. in length. The limbs of the thymus-fed animals averaged in length about the same as the controls; this was true also of body length, as a glance at Table VIII. will show.

In regard to pigmentation, activity and mortality among the

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animals of the two groups, practically no differences were observed. The forelimbs of the larvæ had not, as yet, broken through the skin, but showed as slight bulges or swellings under the skin of the pectoral region. The growth of all the larvæ had been retarded, no doubt, by unseasonably chilly weather.

June 10, all of the larvæ of both thymus and liver-fed cultures, on account of their increased size, were removed from their containers and placed in very much larger basins. The new containers were especially constructed sinks, measuring 30 × 18 × 6 inches. Thirty larvæ were placed in each sink, which were so arranged that fresh, well-oxygenated water flowed through them constantly.

By June 20, two of the control larvæ, and one of the thymus-fed group, were in advanced stages of metamorphosis. The forelimbs appeared and the tail was reabsorbed. June 26, these larvæ had completely metamorphosed. From this date on, metamorphic changes appeared in almost all of the larvæ, both of the control and thymus-fed groups. By July 3, six thymus-fed and eight liver-fed larvæ had metamorphosed, and by July 12, most of the larvæ had already metamorphosed, or else were in advanced stages of the process.

The animals were all killed, and their gonads preserved for microscopic examination. Macroscopic examination of the germ glands of both thymus-fed and control animals revealed no observable differences either as regards the size of the gland or proportion of the sexes.

The gonads were fixed in Flemming's fluid and sectioned at a thickness of 7.5 μ. The sections were stained with iron alum hæmatoxylin, counter stained with congo red. Only four of the gonads were examined microscopically, two from thymus-fed and two from control larvæ.

Microscopic examination of the four gonads revealed nothing of interest. Three of the gonads were from female larvæ, and contained young oöcytes undergoing growth. The single male gland contained only spermatogonia and was of normal size. The thymus feeding appeared to have had no effect upon the growth and development of the gonads and the germ cells.

We may say, then, in the light of this experiment, that feeding

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fresh thymus gland to the larvæ of *Rana pipiens* has no effect upon the growth and metamorphosis of these animals, and the gonads appear to be unaffected by such feeding.

PART II. EXPERIMENTS WITH FEEDING THYMUS POWDER.

The following experiment was started May 3, 1915: The larvæ of *Rana pipiens* were used for the first half of the work. These larvæ had been reared from the egg in the laboratory and were all from one batch of eggs. The feeding work was begun when the tadpoles averaged 28 mm. in length. Most of the larvæ had tiny limb buds of the posterior extremities, at this time, though, as yet, the buds had not differentiated into their two primary divisions. Two series of animals were used, one for feeding work, the other for controls. Both groups were kept under uniform conditions of temperature, light, and water supply. Finger bowls were used as containers, five larvæ to a bowl. Tap water was used to cover them and was changed daily.

The animals of the control group were, each day, fed fresh water algæ, and dried flour paste. This same food was also given to the thymus-fed larvæ, though in smaller amounts in order to obviate any possibility of inanition.

The thymus used was the desiccated glands of sheep prepared by Armour & Co. This powder was mixed with wheat flour in the proportions of three grams of thymus to five grams of flour, enough water was added to make a thick paste which was allowed to dry at room temperature. Pieces of the dried thymus paste about the size of one's thumb nail were finely crumbled and fed to the larvæ. The mixture seemed palatable to the tadpoles for they ate it greedily. The same amount of paste was given each day.

The control animals grew rapidly, and in a few days the limb buds had differentiated into small legs. The development of the thymus-fed larvæ was somewhat slower and, in regard to length, were two or three millimeters behind their controls. On the whole, however, growth in the two series of larvæ was approximately equal, when the fact is considered that the thymus-fed larvæ received less algæ than the controls.

The limbs of the thymus-fed tadpoles differentiated somewhat

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more slowly than the limbs of the controls, and appeared to be smaller in size. There were no observable differences in the pigmentation of the two groups.

This series of larvæ was kept until June 3, just one month from the date the thymus feeding began. They were measured on this date, the control larvæ averaging 39.5 mm., the thymus-fed larvæ 37.5. The tadpoles were then killed and preserved for miscroscopic examination. It is evident that the thymus feeding did not accelerate growth in the animals of this group. The two millimeters difference between the average length of the control group and the average length of the thymus-fed group is, in animals so variable in their growth capacities as frog larvæ, negligible.

On May 9, a second series of frog larvæ were started upon the thymus diet, appropriately controlled. The controls and thymus-fed larvæ of this series averaged 35 mm. All of the larvæ had hind legs though they were very small, and in most of them the toe points were just appearing. These animals were fed the same kind, and amount of food, as the first series received.

The thymus feeding continued for eighteen days, during this period both controls and thymus-fed larvæ grew rapidly, but as recorded for the first series, the controls differentiated limbs faster.

The length of the two groups of tadpoles was approximately equal, both groups averaging 41.5 mm. eighteen days after the experiment began.

Two other series of larvæ were started later, but the tadpoles were too far along in metamorphosis. No results worth recording were obtained.

August, 1915, I obtained some larvæ of the common bull-frog, *Rana catesbiana*. When found, these tadpoles averaged 32 mm. in length. All except six were used for another experiment. The six remaining larvæ were divided into lots of three each, one for controls, the other for thymus feeding. The nature of the food, and amount fed was the same as that recorded for earlier experiments.

For twenty-one days, this series of tadpoles was fed thymus, and at the end of that period, there was no appreciable difference

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between the controls and thymus-fed animals either in regard to body length, shape or stage of development. Before the experiment was discontinued the larvæ of both groups of animals were measured. The controls measured in length 41 mm., the thymus-fed animals 39.5 mm.

In this series of larvæ, as in the two previous series, thymus feeding certainly did not accelerate growth.

On November 1, another set of *Rana catesbiana* larvæ were obtained, and all larvæ of 75 mm. in length were selected for thymus feeding. There were twelve 75 mm. larvæ. They were divided into two groups of six each for controls and thymus feeding purposes. The diet of both groups was similar to that given to the larvæ of the previous series.

November 10 the animals of both control and thymus-fed groups were measured. The controls averaged 83 mm. in length; the thymus-fed larvæ 85 mm. The limb buds of both groups were very small. No other differences in the two groups were noticed.

December 9, the larvæ of both groups were again measured. The controls averaged 89.5 mm. The thymus-fed larvæ averaged 90.5 mm. Pigmentation, body shape, were the same in the animals of both groups.

December 23, the animals of both control and thymus-fed groups were again measured. The controls averaged in length 92.5 mm., the thymus-fed larvæ 93 mm. The limb buds of both groups appeared to be of about the same size and stage of development.

The experiment was discontinued at this time.

It was observed in this series that the thymus-fed larvæ averaged slightly more in length than the controls. The differences, however, are very small.

A last series of feeding experiments with powdered thymus was started November 17, 1915. Eighty small tadpoles (species not determined), averaging 17 mm. in length and without limb buds, were brought to the laboratory on this date. Thirty were selected and treated similarly to those of the earlier experiments. These animals were fed thymus from November 17 until December 7. Upon this date the tadpoles of both control and thymus-

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fed groups were measured. The controls averaged 29.5 mm., the thymus-fed larvæ 30 mm. The disparity in the average lengths of the two series is negligible. The fact to be noticed is that both experimental and control larvæ grew considerably and that both groups possessed tiny limb buds on December 7, when the experiment was discontinued.

Summary of Thymus Powder Experiment.

To sum up the results obtained in the experiments just recorded it may be said that feeding powdered thymus glands to amphibian larvæ of the species *Rana pipiens*, *Rana catesbiana* and *Rana* ——— (species undetermined) does not accelerate growth beyond the normal rate. Some of the observations recorded do, however, indicate that thymus feeding may have a slight inhibitory effect upon limb development. However, in animals so extremely variable in their growth capacities as frog larvæ, to attribute such a slight retardation in limb development to the effects of thymus feeding would be rather unsafe. Aside from this extremely slight retardation in limb development of the thymus-fed larvæ, no results were obtained which would indicate that the feeding of powdered thymus gland to amphibian larvæ has any effect upon growth.

Summary and Conclusion.

To sum up the results of this experiment, feeding thymus gland, either in the fresh state or in the powdered form, to frog larvæ of the species *Rana pipiens*, *catesbiana*, and one undetermined species, does not accelerate the growth processes, or retard the metamorphosis of these animals. The tadpoles seem to develop normally in every respect. The gonads do not appear to be effected by thymus feeding.

These results are at variance with the findings of Gudernatsch. It may be, however, that the species of frog used by this investigator reacts differently to thymus feeding than does *Rana pipiens* and *catesbiana*, thus accounting for the disparity of the results obtained by this investigator and myself.

One point, however, must not be overlooked in this connection, *i. e.*, that the variability of the growth rate in frog larvæ is

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very great, even in larvæ reared from the same batch of eggs and under similar conditions. Very slight environmental changes are sufficient to bring about fluctuations of the processes of growth in these animals, and even when the changes are reduced to a minimum individual variations are great. I have seen tadpoles of the same age, from the same batch of eggs, reared in the same bowl, that varied as much as 20 mm. in length in five weeks' time. I quote this for what it may be worth, but it serves to indicate the complexity of the factors governing the growth of amphibian larvæ. I cannot but think that the results obtained by Professor Gudernatsch are attributable, in part at least, to some other factor besides thymus feeding.

In regard to the feeding experiments with thymus gland, either in the fresh state or powdered form, one point should not be overlooked, viz., that the growth-accelerating principles of the gland, if there be such, may undergo disintegration or suffer a change by contact with the digestive enzymes in the alimentary tract. Feeding experiments with thyroid glands show that no such changes occur, but possibly the same is not true of the thymus.

In conclusion, I wish to acknowledge my indebtedness to Dr. B. M. Allen, the University of Kansas, for the time and encouragement he has given me in my work.

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BIOLOGICAL BULLETIN

THE FORMATION OF STRUCTURES RESEMBLING ORGANIC GROWTHS BY MEANS OF ELECTROLYTIC LOCAL ACTION IN METALS, AND THE GENERAL PHYSIOLOGICAL SIGNIFICANCE AND CONTROL OF THIS TYPE OF ACTION.[1]

RALPH S. LILLIE.

I. Introduction.

In a recent paper on the nature of the physico-chemical processes underlying the conduction of stimuli in living cells[2] I have called attention to the existence of various significant parallels between the transmission of the effects of local chemical or other alteration in metals and the transmission of physiological influence in organisms. These parallels suggest that processes of a kind related to electrolysis may be concerned in the physiological type of transmission, since such local action in metals (*e. g.*, rusting in iron) is now recognized as a phenomenon of electrolysis, due to the formation of local electric couples between adjoining regions of the surface which differ in composition or solution-tension. In particular the rapidity with which the chemical and other effects at the immediate site of action are transmitted to remote resting regions, in rapidly conducting tissues like nerve, indicates that any process involving an actual transfer of material from active to inactive regions cannot possibly form the basis of the transmission. Some influence of an essentially different kind is indicated; and the type of phenomenon long known to electrochemists as "chemical action at a distance"[3]—in which a chemical change at one elec-

[1] From the Laboratory of General Physiology, Clark University.

[2] *Amer. Journ. Physiol.*, 1916, Vol. 41, p. 126.

[3] *Cf.* Ostwald's article, "Chemische Fernwirkung," *Zeitschr. physik. Chem.*, 1891, Vol. 9, p. 540.

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trode of an electrolytic cell instantly calls forth a corresponding change at the other electrode—seems to furnish the closest physico-chemical analogy. In this chemical "distance-action" the transmission is directly dependent upon electrolysis—*i. e.*, chemical change at the contact of electrode and electrolyte, conditioned by the passing of an electric current between the electrodes. The possibility that a process of essentially this type may occur in living cells is not so remote as might seem at first sight, even though conductors of the first class are not present. In many respects the conditions at the boundary between the living cell and its medium are similar to those at the boundary between a metallic electrode and the adjoining electrolyte. Cell-surfaces are electrically polarized; this polarization is subject to change under a variety of conditions; hence local circuits readily arise in cells and nerve-fibers as a result of stimulation or local alteration or injury. These circuits resemble in various essential respects those arising when dissimilar metals in metallic connection are placed in contact with an electrolyte (the usual battery arrangement); thus (1) the potentials can be summed,[1] (2) the potentials vary with the concentration of the electrolyte in essentially the same manner as in the case of metallic electrodes,[2] and (3) a marked degree of polarizability is shown. The inference that chemical changes of the nature of electrolysis may occur where currents enter and leave the cell-surface would seem to be justified by these resemblances. The existence of such a condition would go far to explain many hitherto obscure types of physiological transmission, besides the conduction of stimuli—for example, the transmission of formative or integrative influence in the phenomena of regeneration, form-regulation, growth and development. The general influence of electric currents upon cellular processes would also appear in a clearer light; it is well known that formative and other metabolic processes, as well as activities like contraction and nerve-conduction, are profoundly influenced by such currents. There are also grounds for believing that in the restoration of the normal resting condition of cells, after

[1] *Cf.* Brünings, *Pflüger's Archiv*, 1903, Vol. 98, p. 241.

[2] Macdonald, *Proc. Roy. Soc.*, 1900, Vol. 67, p. 325. *Cf.* also Loeb and Beutner, *Biochem. Zeitschr.*, 1912, Vol. 41, p. 3.

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any kind of activity involving breakdown of material, a process of chemical and structural reconstitution or reorganization takes place, and that in this process electrical factors play an important part.[1] Finally it should be noted that the physiological effects at anode and cathode are typically contrasted in a manner which inevitably recalls the contrast between the two electrodes in electrolysis; and while this resemblance does not prove that processes of electrolysis enter in producing the physiological effect—since the current may have other kinds of polar action—it undoubtedly favors such an interpretation.

The similarity of certain types of electrolytic deposit to vegetative growths has long attracted the attention of chemists. This is especially the case with the so-called lead-trees, tin-trees, etc., obtained when salts of these metals undergo spontaneous electrolysis in contact with baser metals; thus when a piece of zinc is suspended in a solution of tin chloride or lead acetate the metal of the salt is deposited upon the zinc in a tree-like branching form; this metallic deposit grows outward through the solution because each portion of metal as deposited forms a cathodic surface which itself becomes the site of a chemical change similar to that at the original surface of deposition. An analogy to organic growth is evident here; in both processes certain substances are selectively removed from solution, chemically transformed, and deposited to form a definite type of structure. A further resemblance between such a process of electrolytic accretion and true organic growth is that in electrolysis material may be deposited from solutions of *all* degrees of concentration. In this respect the extension of a metallic cathode in a solution of its salt presents a closer analogy to organic growth than does the increase in size of a crystal in a supersaturated solution, since in organic growth the abstraction of food-substances from solution and their transformation into structural material or living protoplasm may also take place from very dilute solution.

Structures having a remarkably close superficial resemblance to plant growths and other cell-structures are also formed under certain conditions (partly described below) from metals under-

[1] *Cf.* my recent paper in *Amer. Journ. Physiol.*, 1917, Vol. 43, p. 43; see pp. 56–7.

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going local chemical or electrolytic change in which insoluble precipitates are formed. The rusting of iron is an example of this general type of phenomenon, although here the deposits of oxide, beyond showing a tendency to spread, *i. e.*, to act as centers for the deposit of new oxide, do not as a rule show structural peculiarities definitely suggesting an organic type of growth. Certain other inorganic precipitates, however, such as the ferricyanides of the heavy metals, especially when formed in the presence of protective colloids like gelatine and egg-albumin, may be deposited in a filamentous or vesicular form and give rise to structures which are strikingly similar, both in morphological constitution and in the conditions of their formation, to definite cellular or other organic growths. In the present paper I propose to describe a number of methods by which these structures may be readily formed; and also certain methods of accelerating or inhibiting their rate of formation, which appear to be closely analogous in their fundamental features to the conditions controlling growth-processes in plants and animals. Since these artificial growth-structures are beyond any question produced by local processes of electrolysis, it seems justifiable to regard the various parallels described below as affording strong support to the above view that processes of a similar nature are intimately concerned in the formation and deposition of structural and other materials in living organisms.

II. Experimental.

The formation of filamentous growths from an electrolytically formed precipitate may be most readily demonstrated as follows. A small piece of ordinary iron, *e. g.*, a nail or tack or piece of wire, is placed in a 2 per cent. egg-albumin solution (fresh egg-white diluted six times and filtered) containing 2 per cent. potassium ferricyanide (K_3FeCy_6). Within a few seconds the blue precipitate of ferrous ferricyanide is seen to form locally at various spots on the surface of the metal; the precipitate is deposited chiefly in the form of slender filaments which rapidly increase in number and length until within fifteen to thirty minutes the entire surface of the metal is covered with a blue-green filamentous hypha-like growth. After a few hours some

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of the thicker filaments may be several centimeters long, usually extending in a straight or sinuous course over the surface of the solution. They are hollow, unbranched, of smooth contour, and exhibit considerable coherence and flexibility; these properties, however, as well as the rate of growth, vary with the content of the solution in albumin and ferricyanide. The rate of formation also varies with the character of the metal, being in general slower with pure than impure specimens of iron; it is also greatly influenced by the contact of other metals, as will be described in detail below.

The actual process of formation presents a striking spectacle under low powers of the microscope. Within a few seconds the precipitate appears upon the surface of the metal in scattered regions, at first as minute rounded vesicles each enclosed by a thin precipitation-membrane; slender cilia-like filaments then push out rapidly into the solution; these steadily elongate, many reaching in a few seconds a length of 50 microns or more. In most cases growth ceases abruptly at a length of a small fraction of a millimeter; this is usually the case with the more slender formations; the broader filaments may continue to elongate by terminal growth until they reach in many cases a length of several centimeters. At their earliest appearance the filaments strongly suggest organic growths like cilia, and what is still more remarkable is the fact that they often exhibit trembling or wavy or in some cases quite regularly rhythmical to-and-fro vibratory movements. This phenomenon is also observed with the similar filamentous growths of zinc and copper ferricyanide, especially the latter, and will be considered further below. Since in living cells cilia typically arise from locally modified regions of the surface protoplasm ("basal bodies") which serve as centers of rhythmical activity as well as of growth, this behavior of newly formed precipitation-filaments increases the impression that the conditions of formation of these artificial structures are of the same fundamental nature as those of actual cell-structures.

Other metals which form insoluble ferricyanides may give rise in the above solution to filaments of a similar type, differing characteristically in their structure, appearance, and rate of formation from those of iron ferricyanide. Small pieces of zinc

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form highly characteristic structures of this kind, especially if in contact with carbon or a nobler metal (Ag, Hg, Pt, Cu, Fe). Pieces of pure zinc, without other metal, form filaments in the above solution much more slowly than iron, presumably because of the more homogeneous structure of zinc and the relative absence of regions differing sufficiently in solution-tension (and hence in potential) to form efficient local couples; but when in contact with a noble metal the rate of formation is rapid. Copper in contact with platinum or carbon also forms filaments in ferricyanide solutions containing sodium chloride in sufficient quantity; for reasons that will appear below, the presence of this or a similar salt is necessary to the process. On the other hand, certain metals which readily form insoluble ferricyanides do not deposit the precipitate in filamentous form; this is true of aluminium, lead, tin, and (in part) manganese. Pieces of aluminium and lead in contact with copper or platinum wire form precipitates rapidly in the above solution; but the deposit merely collects in the form of more or less coherent or vesicular masses at various regions of the surface without forming filaments. Apparently only those metals whose ferricyanides form semi-permeable precipitation-membranes can form filaments. Under the above conditions tin forms a slight and non-coherent precipitate; with manganese some formation of filaments is usually observed, but most of the precipitate is deposited in amorphous form. The following observations have been made chiefly with filaments of iron, zinc, and copper ferricyanide. The more minute structural details of these formations will not be described at length in the present paper,—both in order to save space and also because the experiments themselves are so simple and the materials so accessible that anyone can readily make these observations for himself. The following description will therefore be confined to the more general features of structure and the essential conditions of formation.

Conditions of Formation of Filaments.—In pure solutions of K_3FeCy_6 (2 to 4 per cent. in distilled water) the formation of precipitate is slight and gradual. Pieces of iron or zinc remain for hours in such a solution without perceptible change. The addition of a little soluble alkali salt, however, *e. g.*, NaCl, at

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once leads to a rapid formation of precipitate. The growth of filaments in gelatine or egg-white solutions containing K_3FeCy_6 is in fact dependent upon the inorganic salts associated with the protein. Apparently salts must be present whose anions form soluble compounds with the filament-forming metal; the reason for this requirement will become clear when the process of filament-formation is described in detail. The following experiment shows that the rate of filament-formation varies directly with the proportion of chloride (or similar salt) present in the solution of ferricyanide. A small iron nail (one centimeter long) was placed in each member of a series of solutions of 4 per cent. K_3FeCy_6 containing progressively decreasing proportions of NaCl, viz., 4, 2, 1 per cent., etc., down to 1/128 per cent. An abundant precipitate of iron ferricyanide was formed within ten minutes or less in all solutions containing 1/16 per cent. NaCl or more, and the more rapidly the higher the proportion of NaCl. With 1/128 per cent. NaCl the precipitate formed gradually; while in the entire absence of NaCl none was perceptible after several hours. Similar experiments with pieces of zinc in contact with copper wire gave the same result.

The presence of egg-albumin or gelatine, *i. e.*, an emulsoid colloid capable of having a "protective" action upon finely divided insoluble material, greatly favors the formation of coherent filaments from iron and zinc; with copper this condition is less important. Thus in a solution of 1 per cent. K_3FeCy_6 in distilled water containing a trace of HCl ($n/200$–$n/400$) an iron nail rapidly forms precipitate, but this is largely separated in the form of a loose non-coherent deposit and only partly as filaments. In a similar solution containing 2 per cent. egg-albumin relatively little amorphous precipitate is formed, and the filaments are more numerous, regular, and coherent. Similar conditions hold to an even greater degree for the formation of zinc filaments; in simple aqueous solution the zinc ferricyanide is deposited chiefly in amorphous form or in the form of coarse, brittle, and irregular tubular structures; while with protein present an abundance of slender hypha-like filaments results. This type of phenomenon is undoubtedly of much biological interest. In the absence of the protective colloid the crystals of

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precipitate unite as they separate out to form coarser aggregates of relatively slight coherence; while in its presence each particle of precipitate is apparently at once coated with an adsorbed film of modified protein which serves to bind the particles together and favors the formation of delicate and regular structures. In organisms the deposition of inorganic precipitates like calcium carbonate or phosphate in the formation of skeletal structures (bone, shell, etc.) takes place under essentially similar conditions; the particles are thus deposited in finely divided form, and their arrangement to form definite and regular structures is facilitated. This influence of albumin or gelatine upon the character of precipitation-membranes and similar structures has been investigated by Quincke;[1] he also finds that the colloid modifies very essentially the character of the structures formed.

A certain minimal concentration of K_3FeCy_6 is also necessary for the formation of coherent filaments. In lower concentrations the precipitate tends to separate out in non-coherent form, producing irregular or amorphous deposits. Thus in experiments with iron nails placed in a series of solutions containing 2 per cent. egg-albumin and 1 per cent. NaCl, with varying concentrations of K_3FeCy_6, it was found that while from a 0.8 per cent. solution of K_3FeCy_6 the precipitate separated chiefly in filamentous form, from a 0.4 per cent. solution it was deposited only in small part as filaments but chiefly as an amorphous mass; in 0.2 per cent. very few filaments were formed, and none in more dilute solutions. The same general result was found in a second similar series with a lower concentration of NaCl (1/8 per cent.). Similarly while zinc readily forms filaments in an albumin solution containing 0.25 per cent. K_3FeCy_6, in a 0.13 per cent. solution only a few filaments are formed. In order that coherent structures should be formed the concentration of soluble membrane-forming salt must exceed a certain well-defined minimum.

Structure and Manner of Formation of Filaments.—The precise structure of the filaments varies according to the nature of the metal, the presence or absence of protective colloids, and the concentration of the ferricyanide and other salts present. The

[1] Quincke: *Annalen d. Physik*, 1902, 4te F., Vol. 7, p. 646.

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larger filaments are typically hollow or tubular; it is only the shorter and slenderer structures that appear to be solid, and even here the case is doubtful, since filaments that appear solid on ordinary examination often show a distinct double contour when examined more closely. The probability is that all filaments are hollow, at least when first formed, and that each consists essentially of a solution containing a salt of the metal and enclosed by a semi-permeable precipitation-membrane. The mode of formation about to be described indicates this clearly. The appearance of the mass of filaments covering a piece of metal that has lain for some time in the solution is strikingly vegetative or fungoid in character. The special character or "habit" of the growth varies with the different metals; in the case of iron and copper the filaments are usually more uniform in appearance and have a smoother contour and straighter course than in the case of zinc; with the latter metal the growths are more irregular and varied and have a characteristic granular appearance due to adhering coarser particles of precipitate. The appearance of such growths is often remarkably hypha-like in character; thus a thin strip of zinc about two centimeters long with a copper wire bound about one end, placed in a watch-glass containing a 2 per cent. solution of K_3FeCy_6 in dilute egg-white, developed in the course of eighteen hours a most complicated feltwork of winding and interlacing hypha-like filaments, covering the whole strip from end to end. Numerous tubular filaments extended to the surface of the solution and along the surface, in some cases for three or four centimeters; many such surface-filaments had undergone curious modifications suggesting efflorescences or spore-capsules, *e. g.*, becoming enlarged at their terminations to form stalked vesicular or bladder-like formations with thin walls. Such a mass of modified filaments irresistibly suggests a vegetative growth.

Study of the mode of formation of these tubular filaments under the microscope reveals a somewhat unexpected complexity of conditions. Elongation is not by basal growth, *i. e.*, by deposition of precipitate at the surface of the metal where the metallic ions enter solution, but is always *terminal*, the tube advancing through the solution by the deposition of precipitate

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at its extremity; this process is accompanied by a flow of solution outward through the tube. Filaments of iron ferricyanide show most clearly the nature of the whole process. In a 2 per cent. solution of K_3FeCy_6 in dilute egg-white containing a little NaCl (0.25 per cent.) large hollow filaments grow out rapidly from the metal within a few seconds. Each such filament advances into the solution at an apparently rapid rate (of a millimeter or more per minute) by a uniform motion; its contour is smooth and regular and its shape cylindrical or slightly tapering; it usually follows a straight course, but is easily diverted by encountering obstacles, such as solid particles or other filaments, or by shaking the watch-glass so as to make irregular the deposition of precipitate at the extremity. In a growing filament this extremity is always open, and from the orifice a stream of liquid containing fine suspended particles flows continually outwards for a short distance in advance, suggesting somewhat the smoke emerging from a chimney; this stream of finely divided material leaves behind and on either side a trail of particles through which the growing tubule advances; while at the edges of the open orifice a dense uniform layer of precipitate is continually being deposited in continuation of that already laid down. Growth proceeds rapidly in this manner until some condition arrests the outflow of fluid; this may happen as a direct result of increasing length, the flow through the long capillary tube being slowed more and more by frictional resistance as the length increases, until finally the end is sealed by the precipitate. Evidently a wide tube will grow longer, under these conditions, than a narrow one, and in fact the great majority of filaments cease growth at a length of one or two millimeters or less; it is only the exceptional wider tubes that reach the length of a centimeter or more.

From these facts it is clear that elongation is dependent upon the maintenance of a flow of solution from the metal along the tube. The precipitation at the open end shows that this solution contains a dissolved salt of the metal. Apparently the process takes place somewhat as follows. At the anodic portions of the metallic surface ferrous ions enter solution; these form with the ferricyanide ions of the solution already present a precipitate of

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ferrous ferricyanide which is deposited as an incomplete vesicle or ring of semipermeable membrane forming the beginning of the tube; ferrous ions continue to be given off from the iron surface, forming ferrous chloride with the chlorine ions present; the interior of the tube thus contains a solution of ferrous salt which is continually being propelled forward through the open extremity, and there meeting ferricyanide from the external solution forms the precipitate.

The question of what maintains the flow is interesting. One possibility is that each ferrous ion is hydrated, *i. e.*, carries water along with it as it enters solution; water thus flows in the same direction as the ferrous ions, the energy of the flow being derived from the solution-pressure of the iron. Other factors, however, probably enter; it is especially to be noted that the point of origin of each tube represents an anodic area, from which the positive electrical stream enters the solution, reëntering the metal at some cathodic region or regions outside of the tube. An electric current thus passes outward along the tube; and this must cause effects of the kind always found when such a current traverses a system of capillary tubes containing a solution, *e. g.*, a porous partition; these effects are of the kind known as "electrical endosmose";[1] when the substance of the capillary wall is negatively charged the adjacent solution is positive and travels with the positive stream. It is known that iron ferricyanide particles are charged *negatively* in contact with water;[2] hence the layer of solution at the walls of a tube consisting of this material must be *positively* charged and undergo transport in the direction of the positive stream. The flow of solution through the tube is thus probably in large part due to electrical transport by the current of the local circuit. One necessary factor in the process of formation of the tube is the semi-permeability of its wall, which permits water but not salts to penetrate; the solution of ferrous salt thus transported along the tube can

[1] For a description of this phenomenon see the larger textbooks of physics; a good account of the essential conditions is given in Höber's "Physikalische Chemie der Zelle und der Gewebe," 1914, pp. 234 *seq.*

[2] The ferrocyanides and the ferricyanides of the heavy metals form negatively charged particles. The potential-difference of Prussian blue against water is .056 volt. (*Cf.* Burton, "The Physical Properties of Colloidal Solutions," London, 1916, p. 135.)

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form precipitate only where it flows out at the open extremity and meets the ferricyanide outside; part of the precipitate is scattered outside the tube, as already described, or remains finely suspended within its interior; but part adheres at the outlet and contributes to the growth of the tube.

According to this conception of the tube-forming process, the rate of flow of fluid along the tube must vary directly with the intensity of the electric current through the tube—*i. e.* (assuming constant E.M.F.), with the electrical conductivity of the solution within the tube. This explains why the rate of growth of the filaments varies directly with the concentration of NaCl (or similar salt) in the ferricyanide solution. The only salt that can remain in solution inside the tube and contribute to the conductivity of its contents must obviously be one that is not precipitated by the ions of the tube-forming metal.

Structure of Filaments.—The structure of a tubule is partly determined by the nature of the material composing it, partly by the conditions under which the material is laid down, such as the size of the anodic area, concentration of salts, presence of protective colloid, external mechanical influences, etc. In general the consistency of the precipitate is coarsest in the case of zinc, and finest in the case of copper; hence tubes of zinc ferricyanide have a coarsely granular aspect and are more irregular in form, while those of iron and copper have smoother contours and a more uniform appearance. There is always considerable variation in the diameters of the tubes formed in any single experiment; in general an increase in the concentration of NaCl in the solution (*i. e.*, more rapid rate of formation) favors the production of wider tubes. Apart from differences in caliber there is little variation in the structure of those tubes which lie entirely below the surface of the solution; typically these are cylindrical or slightly tapering in shape, and follow a more or less tortuous course, varying according to the number and nature of the mechanical obstacles encountered during their formation; zinc filaments show more irregularities of this kind than iron or copper filaments. Those filaments which reach the surface of the solution during their formation undergo various characteristic modifications; thus vesicular or bladder-like thin-walled structures may

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be formed, especially in the case of zinc, as above described; several such structures derived from different filaments may fuse and form a compound vesicular or cellular mass. One of the most interesting modifications is of a kind often shown by surface-filaments of iron or copper ferricyanide, consisting in a series of transverse striations resembling those of a striated muscle fiber. A regular ladder-like structure of this kind may extend for a long distance; it is apparently due to the presence of alternately denser and thinner zones in the precipitation-membrane forming the wall of the tube. This structure is found only in the larger filaments running along the surface of the solution, and indicates that the deposition of precipitate takes place in an intermittent manner under these conditions; the regular rhythmical recurrence of a metastable condition of some kind, such as supersaturation—as in the phenomenon of the Liesegang rings[1]—may determine its production, but the precise conditions require further study. Occasionally the finely divided precipitate inside the tube may aggregate to form rounded masses repeated at regular intervals, in a manner suggestive of a row of spores.[2] Filaments of zinc ferricyanide may at times exhibit a regularly constricted or moniliform appearance, but they never form regular cross-striations like those shown by iron and copper filaments; the coarser texture of the precipitate is probably responsible for this difference.

Certain other types of precipitation-structure should also be mentioned briefly.[3] Masses of precipitate of a vesicular or chambered rather than filamentous structure are often laid down at the surface of the metal; this is more frequently the case with zinc than with iron or copper. Apparently in such cases the walls of the first formed vesicles remain intact, and do not rupture to allow the solution of metallic salt to flow out in the continuous

[1] *Cf.* Liesegang, "Beiträge zu einer Kolloidchemie des Lebens," Dresden, 1909. For a more recent study of this phenomenon *cf.* Stansfield, *American Journal of Science*, 1917, Vol. 43, p. 1.

[2] The structure of these tubules resembles closely that of the "osmotic stems" described and pictured by Leduc in his "Mechanism of Life," London, 1911, pp. 141–2.

[3] In Lehmann's "Molekularphysik" various precipitation-formations are compared with different types of organic structure, and much interesting detail is given.

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stream necessary for the formation of filaments. Once formed, these precipitation-vesicles may be displaced or detached by the pressure of others formed later, and large groups or heaps of such structures may thus collect. In the case of copper one highly characteristic type of formation, frequently found in solutions of K_3FeCy_6 containing considerable chloride (*e. g.*, 4 per cent. NaCl *plus* 2 per cent. K_3FeCy_6), is a group of slender tapering filaments grouped radially about a central vesicular mass of precipitate, the whole structure suggesting a miniature chestnut-burr.

It should be noted that these various types of structure, together with many others, are produced—as is also the case with the majority of living structures—by combinations of microscopic hollow elements, vesicular or tubular in form, with walls consisting of semi-permeable membranes. The above resemblances to organic growths are undoubtedly dependent upon this fundamental similarity of physico-chemical constitution.

Influence of Contact of other Metals upon Filament-formation.—Before proceeding to describe observations showing the inhibiting and reinforcing influence which the contact of one metal may exert upon the formation of filaments from another, the general theory of this effect ought briefly to be considered. As already explained, the formation and growth of these structures depend upon a process of electrolytic local action or electrolysis; the region from which the filament grows out represents an anodic area, *i. e.*, an area where ions of the membrane-forming metal enter solution; these ions then interact with the ferricyanide ions to form the precipitate; to each such anodic area corresponds a cathodic area (or areas) at some other region of the metallic surface, where the positive stream reënters the metal to complete the circuit; at this area hydrogen ions are deionized and hydrogen gas is freed with the formation of alkali. In the case of iron these areas may readily be demonstrated by the addition of a little phenol phthaleïn to the ferricyanide solution (as in the ferroxyl reagent of Walker);[1] the cathodic regions, which typically remain bright and free from filaments for some time, then

[1] *Cf.* Walker, Cederholm and Bent, *Journ. Amer. Chem. Soc.*, 1907, Vol. 29, p. 1251.

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exhibit the characteristic red color of the indicator in alkaline solution. Each filament thus originates from an anodic area in a local electric circuit; the precise mechanism of the formative process has already been considered.

Specimens of ordinary iron always exhibit a large number of local couples of this kind, because of the characteristic lack of electro-chemical homogeneity of this metal. As has frequently been pointed out, this condition explains the susceptibility of iron—especially impure iron—to rust; an iron surface is composed of numerous areas which differ in physical condition and hence in electrolytic solution-tension; areas of higher solution-tension form local anodes, and rusting is a consequence of electrolysis at these local circuits. Iron prepared in as high a state of purity as possible is surprisingly resistant to corrosion.[1] What is true of rusting is true also of the formation of precipitates with electrolytes like ferricyanide; filaments form more slowly from chemically pure iron wire than from pieces of ordinary commercial metal (nails, etc.). Zinc and aluminium, although metals of higher solution-tension than iron, are more readily obtained in a relatively homogeneous condition; hence they resist corrosion better and form precipitate more slowly in ferricyanide solutions.

Such considerations explain the accelerating influence which the contact of a nobler metal, like platinum or copper, has upon the formation of precipitation-filaments from zinc, iron, or copper. The nobler metal (that of lower solution-tension) has a higher potential in contact with the solution than the less noble; hence a circuit is formed in which the latter acts as anode, *i. e.*, enters solution as cations which form precipitate with the ferricyanide. Conversely, a less noble metal, *e. g.*, magnesium or manganese in relation to iron or zinc, *inhibits* the formation of filaments from the membrane-forming metal. Both the reinforcing and the inhibiting influences have very pronounced and evident effects, which are perceptible not only at the immediate region of contact of the two metals, but for a considerable distance, *e. g.*, of several centimeters, beyond this region. The degree of this influence at any point on the surface of the filament-forming

[1] For references to the literature in this field see my paper above cited, *Amer. Journ. Physiol.*, 1916, Vol. 41, p. 126.

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metal depends in general upon three chief factors; these are, (1) the nature of the second metal, (2) its distance from the point under consideration, and (3) the electrical conductivity of the solution. The electromotive force of the local circuit depends primarily upon the difference between the specific solution-tensions of the two metals; hence under otherwise equal conditions zinc is less effective than manganese, and manganese than magnesium, in inhibiting the formation of filaments from an iron surface. The other two factors, (2) the distance between the contact of the metals and the point in question, and (3) the electrical conductivity of the solution, determine the resistance of the portion of the local circuit which includes that point; this determines the intensity of the current and hence the rate of local electrolysis at the point. The effect is thus greatest near the region of contact and diminishes as the distance from this region increases.[1]

The actual phenomena may now be illustrated by the description of typical experiments. In order to show the accelerating influence of a nobler metal, zinc is more satisfactory than iron, for the reason that it can more readily be obtained in comparatively pure and homogeneous condition, in which state it forms precipitate in ferricyanide solutions very slowly unless in contact with the other metal. It is difficult to procure non-reactive specimens of iron; pure iron wire of the kind used in standardizing (*e. g.*, Baker's labeled 99.7 per cent. pure) forms filaments rapidly in a 2 per cent. solution of K_3FeCy_6 in dilute egg-white; the rate of formation is accelerated by encircling the iron with copper or platinum wire, but the degree of acceleration is comparatively slight, and the effect is much less striking than when zinc is used.

Such experiments as the following are easy to perform and invariably give striking results. A small strip, *e. g.*, 2 cm. long by 1 mm. wide, cut from a bar of pure zinc, is encircled at one end with a ring of fine copper wire, and placed in a 2 per cent. solution of K_3FeCy_6 in dilute egg-white. Within a minute or two the characteristic thin-walled transparent vesicles appear

[1] *Cf.* the second paper of my recent series on the physical chemistry of the conduction-process, *Amer. Journ. Physiol.*, 1915, Vol. 37, p. 348.

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at the surface of the metal, and somewhat later slender filaments begin to grow out; in a short time (half an hour or less) the metal is covered with filaments and vesicular formations from one end to the other. The first appearance of these structures is typically near the contact with the copper; a gradient in the rate of formation is thus apparent, with the most active region near the copper. This accelerating influence of the nobler metal is clearly perceptible at the extremity of the strip, *i. e.*, for a distance of some centimeters from the contact. A similar "control" piece of zinc, without copper, shows only a few irregular and slightly developed filaments at a time when the copper-bound piece is completely covered with a luxuriant hypha-like growth. Platinum and iron wires have the same general effect as copper, only with platinum the accelerating influence is distinctly greater, and with iron less, than with copper. Carbon may also form the substance of the cathodic area; a piece of zinc marked with lead-pencil shows a rapid development of filaments, beginning at the marked area and grading off from there.

Striking experiments may also be performed with copper as the filament-forming metal. In this case a higher concentration of NaCl is required for the rapid formation of filaments; beautiful structures develop after some minutes in a solution containing 4 per cent. K_3FeCy_6 and 4 per cent. NaCl (with or without egg-white). A piece of fine copper wire 3 or 4 cm. long, encircled at one end with a platinum wire, or wound around a piece of carbon, shows a highly characteristic development of filaments; some of these within a few hours may reach a length of several centimeters; such filaments, as already described, often exhibit a very perfect and regular cross-striation where they run along the surface of the solution. A similar piece of copper wire without contact of nobler metal or carbon remains unchanged.

Many variants of this type of experiment will readily suggest themselves. Experiments with plated wires yield instructive results. Pieces of "galvanized" (zinc-plated) iron usually show the first development of filaments of zinc ferricyanide near the cut surface where the iron is exposed, and gradients in the rate of formation extending from this region along the wire are frequent. It should be added, however, that these gradients

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are often obscured by irregularities in the rate of action, due presumably to lack of uniformity in the zinc layer (local interruptions, etc.). Platinum wires coated with copper in an electrolytic bath also make interesting demonstrations; such a wire shows no formation of filaments in a solution of K_3FeCy_6 and NaCl if the coating of copper is continuous; but if it is scraped away at any region so as to expose the underlying platinum, the copper near this region soon puts out filaments, and the process extends for a distance of some centimeters from the area of exposure. Such experiments are instructive as exemplifying the nature of the effects that may follow local alteration in the electromotor character of a surface; and they may be used to illustrate by analogy such physiological phenomena as the initiation of growth-processes or regeneration in living organisms by incision, amputation, or similar treatment.

The reverse type of experiment, in which the formation of filaments from one metal is inhibited or prevented by contact with another metal of higher solution-tension, is equally readily performed. Such an effect is illustrated in the just cited experiment with zinc-plated iron wire; the exposed iron surface develops no filaments; their formation is evidently prevented by the adjacent zinc, which forms with the iron a couple in which the zinc is anode; this, it may be added, is the reason why such wires do not rust. The inhibitory influence of zinc upon the formation of ferricyanide filaments from iron may be shown in a more striking manner as follows; a straight piece of thin bright iron wire (*e. g.*, Baker's C. P.) some centimeters long, one end of which is wound about a small strip of zinc, is placed in a 2 per cent. K_3FeCy_6 solution in dilute egg-white. Filaments put forth rapidly from the zinc, especially near the iron, but the iron itself remains perfectly bright and bare, and may show no development of filaments for hours. If then the wire be cut in two by scissors, the part remaining in connection with the zinc remains unchanged, while the isolated part quickly develops the characteristic blue-green filamentous growth of ferrous ferricyanide. Evidently this development had previously been repressed by the influence of the zinc; when the connection is severed the iron reacts as usual. This experiment is even more

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striking if a metal of higher solution-tension than zinc is used, such as aluminium, manganese, or magnesium. Magnesium is especially effective, since the ferricyanide of this metal is soluble and hence does not accumulate at the anodal regions and arrest the inhibitory action by polarization; such an iron wire remains bright until the magnesium is completely dissolved; then filaments grow out as usual. Apparently when zinc is used the accumulation of zinc ferricyanide may interrupt contact between the metals or render the resistance of the local circuit too high (*i. e.*, have a polarizing effect), and by degrees filaments form from the iron. In addition to this effect, contact of the zinc ferricyanide precipitate with the iron appears to form local couples in which the iron acts as anode, so that iron in contact locally with this precipitate usually exhibits in course of time a development of blue filaments. This, however, is an incidental effect due to the formation of another type of circuit. Pieces of aluminium or manganese are almost as effective as magnesium in preventing the formation of iron filaments in the above solution. A large variety of experiments of this type may be performed with iron wire. In a similar manner zinc may be used as the filament-forming metal, and the process inhibited by magnesium, aluminium, or manganese.

As already mentioned, this inhibitory influence may be effective for a distance of several centimeters from the region of contact of the two metals, and a gradient in the intensity of the influence is apparent. After the current of the circuit has decreased, as the result of the accumulation of precipitate or other polarization, an iron wire with zinc connection often begins to send out filaments; these tend to be first formed at the region farthest removed from the zinc. Irregularities, however, are frequently observed in such experiments, due no doubt to local irregularities in the composition and physical condition of the iron. The biological analogies to these effects will be considered later.

An interesting type of effect is seen when an iron wire 3 or 4 cm. long is in contact at one end with a piece of zinc and at the other with copper or platinum. With this arrangement gradients in the formation of iron filaments are very distinct; at first these

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filaments are formed only in the immediate neighborhood of the copper and are entirely absent near the zinc. Copper and zinc influence the reaction of the iron wire with the solution in opposite manners, the former promoting the formation of iron filaments, the latter inhibiting this process. The zinc and the copper, although separated by the length of iron wire, can be shown to modify each other's action; thus filaments grow out from the zinc under the influence of the iron, but more slowly than in the absence of the copper; similarly the growth of filaments from the iron near the copper is much slower than in the absence of the zinc. The effect is as if the formation of filaments from the zinc inhibits or holds in check the formation of filaments from the iron, and *vice versa*. This mutual interference is obviously due to the intersection of the electrical currents from the two circuits, and resulting compensation-effects. Severing the iron wire between the copper and the zinc removes this influence, with the result that then filaments are put forth rapidly from both iron and zinc; *i. e.*, the process at either filament-forming metal is released from a certain constraint, due to the polarizing influence of the electrical current from the other circuit; it then proceeds more rapidly than before. Physiological analogies to this type of effect readily suggest themselves; the development of shoots in one region of a plant stem inhibits their development in adjoining regions; removal of a rapidly growing shoot may thus cause buds to develop from the axils and elsewhere, which otherwise would have remained dormant.[1] Similar phenomena are seen in hydroids and other animals, and will be discussed more fully below. Physiological "dominance" of this type is often simulated in a suggestive manner by experiments of the above kind. For example, a thin strip of zinc 3 cm. long was in contact at one end with a piece of pure iron wire and at the other end with a piece of magnesium. A well-marked gradient was shown; near the iron filaments were rapidly formed from the zinc, but near the magnesium slightly and slowly. It was noteworthy that the piece of iron wire gradually formed filaments in spite of the contact with the zinc; *i. e.*, the presence of the magnesium

[1] For numerous examples *cf.* McCallum, "Regeneration in Plants," *Botanical Gazette*, 1905, Vol. 40, pp. 97, 241.

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inhibits the protective influence of the zinc upon the iron,—evidently because the zinc forms the cathode in the zinc-magnesium couple, the current from which opposes and partly compensates that from the zinc-iron couple. In another experiment a similar strip of zinc was in contact with platinum wire at one end and with magnesium at the other; in this case the zinc remained free from filaments for a long time; after 18 hours a dense growth of filaments had developed near the platinum; toward the magnesium the growth decreased, and near the contact with this metal the zinc was entirely free from filaments. In a similar manner the contact of iron or zinc with a platinum-bound copper wire inhibits the formation of filaments from the copper. A piece of copper wire 4 cm. long, encircled at one end with platinum wire, was placed in a solution containing 4 per cent. K_3FeCy_6 and 4 per cent. NaCl; filaments and bur-like tufts of copper ferricyanide appeared rapidly. But a similar piece of platinum-bound copper which was placed in contact at the other end with an iron wire showed only slight and gradual formation of precipitate; under these conditions filaments form rapidly from the *iron*, and their formation apparently has the effect of inhibiting their development from the copper. What really happens is that the current of the copper-iron circuit is opposed in direction to that of the copper-platinum circuit and compensates the latter.

Attention should also be called to a type of experiment in which the two precipitate-forming metals are not in direct contact with each other but are connected by a conductor which itself undergoes no chemical change, like platinum, carbon, or copper. For example, a piece of iron and a piece of zinc connected by a stretch of copper wire 1 cm. long were placed in 2 per cent. K_3FeCy_6 in dilute egg-white; the zinc immediately formed filaments, but not the iron. Similar pieces of iron and zinc, each in contact with a separate piece of copper, both form filaments rapidly, as already described; but when the two are in contact with the same conductor the zinc prevents the formation of filaments from the iron in the same manner (though to a less degree) as when the metals are in direct contact. It is further to be noted that under these conditions the iron, though not

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itself forming filaments, retards or partly inhibits their development from the zinc; *i. e.*, zinc in contact with copper alone forms filaments more quickly than when in contact with a copper wire which is itself in contact with iron. This mutual influence is greatest when the two metals are close together; when they are separated by a sufficient stretch of conducting wire the effect becomes inappreciable. Thus with a distance of 3 cm. between the zinc and the iron, the latter metal, as well as the zinc, formed filaments, but more slowly than in the absence of the zinc; the influence of the zinc was perceptible, but insufficient to suppress entirely the action at the iron. Increasing the distance of separation still further renders the influence inappreciable. This experiment recalls such physiological phenomena as the decrease of growth-inhibiting dominance with distance.[1] Experiments with platinum as the connecting metal give similar results; only here, because of the nobler character of the platinum, the distance between the zinc and the iron must be less than when copper wire is used, if the formation of filaments from the iron is to be entirely suppressed. In other words, both zinc and iron have a stronger tendency to form filaments when in contact with platinum than when in contact with copper; hence in order to counteract this tendency in the iron its distance from the zinc must be less.

It will be evident that effects of the above kind have many close biological analogies.[2] Increased physiological or metabolic activity at one region of a living organism often results in the decrease or cessation of activity at another, usually adjoining, region. Perhaps the best known instances are seen in various phenomena of growth and regeneration to which attention already has been called; the reciprocal inhibition of antagonistic neurones in the central nervous system is probably a phenomenon of the same essential type. There is also good reason for associating with this class of phenomena such regular and characteristic physiological processes as the automatic initiation and arrest of activity at any region of a conducting element (*e. g.*,

[1] *Cf.* Child, "Senescence and Rejuvenescence" (especially Chapter 9), and "Individuality in Organisms," for a full account of these phenomena (University of Chicago Press, 1915).

[2] See Section III. below, page 171.

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nerve fiber) as the advancing wave of excitation reaches and passes that point.[1] In the case of these various vital phenomena it seems highly probable, from the degee and nature of the resemblances which they bear to the simple physico-chemical phenomena described in this section, that the essential determining factors are also electrical currents between adjoining cells or cell-regions. These, according to their direction, local density, and intensity, may initiate, inhibit, or reinforce processes at various points along the path of the current.

It may sometimes be desirable to demonstrate still further the electrical nature of these inhibitory and accelerating influences. This may be done most readily by passing the current from a battery, connected with a rheocord, through the ferricyanide solution, using iron wire as electrodes. The formation of filaments at the anode is promoted and at the cathode is prevented, to a degree which varies with the potential between the electrodes; this potential may be changed at will by shifting the position of the slider.

III. Biological Comparison.

In comparing phenomena of the above described kind with physiological phenomena it is obvious that careful distinction must be made between resemblances that are superficial and resemblances that are based upon a fundamental identity in the nature of the determining conditions. It need scarcely be pointed out that in the specific character of the structure-forming chemical reactions there is no resemblance between the two processes under comparison. The complex and imperfectly understood metabolic transformations in living cells are of a kind entirely different from the simple cross-decompositions and precipitations of the above experiments. The resemblances relate to the conditions under which the chemical reactions take place, to the type of structure formed, and to the mode of transmission of chemical influence from one region to another region which is connected with the first only through the medium of an electric conductor. Chemical action at a distance is a phenomenon universally met with in organisms; and the attempts to explain

[1] *Amer. Journ. Physiol.*, 1916, Vol. 41, see pp. 134–5.

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this action on the basis of transport of actual materials—through the flow of sap, blood, or other circulating medium—are in many cases patently inadequate. Consider—to take an extreme instance—the transmission of chemical influence from the brain to a secreting gland-cell; here obviously the problem of the nature of physiological conduction enters; and it is more especially this problem—the nature of transmission of physiological influence where no direct transmission of material is possible—upon which experiments of the above kind appear to throw light. The problem is plainly a most fundamental one, since physiological coördinations of the most varied nature depend upon the rapid conduction of chemical influence between different parts of the organism. The integration of the whole complex system of structures and chemical reactions into a unified and regularly acting living organism probably depends more essentially upon transmission of this type than upon any other single process.

The transmission of formative influences, sometimes of a growth-inhibiting, sometimes of a growth-promoting kind, from one region of the organism to another is a well-known phenomenon in both animals and plants. Usually it has been explained either as due to the production and transport of special materials (organ-forming substances, hormones, etc.), or as due to changes in the distribution of materials already present (changes in the flow of water in plants, increased availability of nutritive substances to one organ when another organ is removed, etc.). In animals a special rôle has at times been assigned to nervous influences; the nervous system[1] or sense-organs[2] have been shown in some cases to influence the rate of regeneration, *i. e.*, a transmissive instead of a transportative means of control[3] has been recognized; but in plants explanations based upon the assumed existence of form-determining

[1] This influence is seen (*e. g.*) in Herbst's experiments in which the regeneration of the eye in Crustacea was found to depend on the presence of the optic ganglion; also in planarians and other forms.

[2] The presence of the marginal sense organs in regenerating pieces of the medusa *Cassiopea* favors regeneration, according to the recent observations of Cary, *Journ. Exper. Zool.*, 1916, Vol. 21, p. 1.

[3] For a discussion of the general nature of this influence see Child's "Individuality in Organisms," especially Chapter 6.

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substances have been most generally employed (Sachs and others).[1]

Among the various phenomena of organic growth and development, regenerative processes seem most evidently open to interpretation in the light of the foregoing experiments. In the regenerating organism growth or development is initiated as a result of the removal of one of its parts, the usual result being the restoration of the missing part or its equivalent. In many cases the part removed is replaced by growth at the cut surface; in other cases the remaining portion of the organism undergoes a more or less extensive reorganization, called by Morgan "morphallaxis"; in still others, especially in plants, removal of an organ initiates the development of a similar organ from another locality, *e. g.*, when the removal of the growing tip induces the development of lateral branches from axillæ or other regions at a distance.

The first class of cases, where growth at the cut surface restores the missing part, may be compared with the case of a membrane-forming metal in a ferricyanide solution, *e. g.*, a piece of iron wire in which the filamentous or membranous growth has accumulated to a degree sufficient to insulate the metallic surface from the surrounding solution, *i. e.*, to arrest the flow of current between anodic and cathodic regions by the interposition of the non-conducting coating of precipitate. If then a cut be made exposing a fresh surface of metal, the formation of precipitate is at once resumed and continues until a new state of equilibrium is established; the accumulation of electrolytically formed structure will then be approximately the same as before. The whole structural system, consisting of the metal with its outer coating of precipitate, will exhibit essentially the same constitution as before. Again in the case of a strip of zinc encircled by a copper wire, as in the experiments described above, the zinc forms the anode of the couple, and its surface is soon covered by precipitation-structures, whose accumulation progressively retards the flow of current and with it the structure-forming electrolysis. When the fresh surface of zinc is exposed, the formation of a new filamentous coating follows simply from the reëstablish-

[1] For an experimental examination and critique of this type of hypothesis, *cf.* the paper of McCallum above cited.

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ment of the electrical circuit. As the current flows, structural material is formed and deposited at the anode, until its accumulation increases the resistance of the circuit and checks the current and hence the electrolysis which produces the structure. Similarly in the case of a regeneration-process where proliferation starts at a cut surface at which previously quiescent cells have been exposed, it is possible that the condition of quiescence is due to insulation by the surrounding cells and other tissue-elements; if these are removed the flow of an electrical current and the formation of new structure are enabled to take place.

The case of morphallaxis is more complex, and is difficult to simulate by a model of the above kind. In this case constructive processes at one area are accompanied by regressive processes at another area; *i. e.*, growth at one region forms the condition of inhibition of growth or of positive regression at other regions; the whole mass of organized living material is thus worked over or reconstituted until a second equilibrium is reached; the character and distribution of structural material is then similar to that existing before mutilation took place. The conditioning of inhibitory processes at one region by active processes at another, and *vice versa*, is a frequent phenomenon in organisms; and analogies are afforded by the experiments cited in the preceding section. In general, in any electrolytic couple the processes at the anode are of an oxidative, those at the cathode of a reducing nature; and it is not difficult to understand how in a living organism increased chemical activity of a certain kind at one region (*e. g.*, oxidation) should result in decreased activity (or activity of the reverse type) at another region related to the first in the manner in which the electrodes of a circuit are related to each other. The inhibition of filament-formation at iron surfaces by the contact of a baser metal illustrates an essentially similar condition. The constant form-relations of an organism or of its parts may thus represent a condition where a complex system or combination of electrolytic processes, interrelated as above, mutually equilibrate one another, such equilibration involving a certain constancy in the relative positions, dimensions, and form-characters of the chief structural elements. This condition would imply a constancy in both the morphological and the

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physiological constitution of the system, which would be regained after disturbance. I introduce this abstract conception of the organic equilibrium in order to suggest a general point of view from which the actual phenomena of form-regulation may perhaps be considered to advantage. There is much evidence indicating that many structural correlations are controlled by electrolytic processes of the above kind.

Morphallaxis is essentially a phenomenon of form-correlation, which is exemplified by the third type of process mentioned above, in which the growth of one part may be promoted or inhibited by growth in another part more or less distant from the first. For example, in plants the development of new buds or roots is prevented so long as the main growing tip or root-system is intact; formative inhibitions of this type are extremely frequent; hence the isolation of an organ, by removing the inhibiting influence of other parts, may cause the development of roots, shoots, or other organs of a kind entirely different from itself, The whole organism may thus be reproduced in certain cases; the leaves of *Begonia* and *Bryophyllum* furnish the most striking instances of this phenomenon. In plants the normal correlation between the growth of different organs appears to depend largely on this influence. In the lower animals various regions or parts of the organism are also capable of reconstituting the whole when removed from the influence of other regions. In many axiate animals the more anterior regions thus "dominate" the more posterior regions, and a gradient of influence exists, as shown in detail by Child in his recent remarkable book.[1] The controlling influence is greatest near the dominant region and decreases with increasing distance, so that a sufficient degree of isolation for independent development may often result from simple increase in length; *e. g.*, in a planarian or a hydroid stem the portion of the animal most distant from the head end or the hydranth may form a new individual while still connected with the parent. According to Child, individuation in either asexual or sexual reproduction consists essentially in the removal of one region of the organism from the growth-inhibiting influence of other regions; the region thus physiologically isolated then

[1] "Senescence and Rejuvenescence," especially Chapter 9. See also the same author's "Individuality in Organisms."

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develops on its own account. Cases of compensatory regulation,[1] *i. e.*, where the rate of regeneration is greater the greater the number of the missing parts, may be understood as due to the more complete removal of the inhibiting influence exerted normally by those parts. In other cases one region may promote or reinforce the growth or development of another region; the nervous system appears to have this relation to regeneration in a number of cases; the presence of sense-organs promotes regeneration in Cassiopæa; the intestinal tract has a similar influence in planarians.[2]

Some of the more suggestive parallels between regenerative or growth processes and the behavior of combinations of filament-forming metals have already been pointed out. The formation of precipitation-filaments from one metal is inhibited by the contact of another metal of higher solution-tension. Hence an iron wire connected with a piece of manganese, aluminium, zinc, or magnesium remains free from filaments for a considerable distance from the region of contact. If a piece of such wire be isolated by cutting, it immediately develops filaments. Similarly iron wire connected with one of these metals through a short copper wire develops no filaments; cutting the connecting wire immediately initiates their formation. These instances are sufficient for illustration of this kind of effect. The precise results obtained vary with the special nature of the conditions, and may be controlled at will by the experimenter.

The reverse class of cases where the formation of filaments is promoted, instead of inhibited, may be illustrated by a strip of zinc in contact at one end with a piece of carbon, platinum, copper or iron. Filaments are formed from all parts of the zinc surface, most rapidly near the region of contact. If then part of the zinc is severed from its connection with the other metal the development of filaments from the isolated part is at once checked; similarly with the whole strip if the platinum or copper is removed. One might compare the influence of the latter

[1] *Cf.*, *e. g.*, Zeleny's work on brittle stars and other forms, *Jour. Exper. Zoöl.*, 1904, Vol. 2, p. 1.

[2] Instances of the influence of the nervous system and sense-organs have been already cited; for the case of the intestinal tract, *cf.* Bardeen, *Amer. Journ. Physiol.*, 1901, Vol. 5, p. 1.

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metal with that of the sense-organs in a regenerating portion of a medusa. The influence of the nucleus in cell-regeneration and cell-growth is another possible parallel of a more general kind,

Cases of reciprocal influence, where two or more different organs exercise mutual control upon one another's growth or development are also frequent in organisms. The development of roots and shoots from the leaves and stem of *Bryophyllum*, as recently described by Loeb,[1] furnishes very clear illustrations of this phenomenon; the growth of a bud on a piece of stem inhibits the growth of roots on an attached leaf; if the bud is removed, roots grow out; similarly roots growing from the stem inhibit the formation of shoots from a leaf. Such cases resemble the above combination where zinc in contact with a copper wire inhibits the development of filaments from iron also in contact with the wire; this influence is also reciprocal, although zinc, because of its greater solution-tension, has the predominant effect.[2] In the inorganic model the direction and intensity of the electrical current flowing between the precipitate-forming metal and the solution, and hence the rate and the character of the structural development, depend upon the special characteristics of the two intersecting circuits. The conditions in the living organism are probably similar in a general sense; and compensations and reinforcements occur, varying according to the character of the connections, the distances and other space-relations, the electrical resistances, etc. Other factors, however, including probably the flow of dissolved materials, almost certainly enter in actual cases of growth and regeneration in organisms. Nevertheless it appears probable that the most

[1] J. Loeb, *Botanical Gazette*, 1915, Vol. 60, p. 249; 1916, Vol. 62, p. 293; 1917, Vol. 63, p. 25.

[2] Loeb enunciates the rule: "If an organ *a* inhibits the regeneration or growth in an organ *b*, the organ *b* often accelerates and favors the regeneration in *a*." (*loc. cit.*, 1915, p. 276). This kind of relationship is also exemplified by the mutual influence of zinc ($= a$) and iron ($= b$) in direct contact with each other. Other well-known general facts of regeneration may be simulated by arrangements of the above kind. Thus the development of a polyp on a tubularian stem inhibits the development of polyps at other regions of the same stem, just as the outgrowth of a shoot on a plant stem inhibits growths from neighboring axils, etc. Similarly the formation of precipitation-filaments from a piece of zinc in contact with a copper wire is checked by the contact of another piece of zinc (or iron, etc.) with the same wire at not too great a distance.

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general and fundamental basis of a reciprocality of this kind is electrical; morphological polarity, a closely related phenomenon, would then have a similar basis,[1] and probably also other cases of reciprocality, like reciprocal inhibition in the nervous system. It is well to recognize, however, that together with this factor others often undoubtedly enter, partly independent, partly dependent upon the electrical factor.[2]

The distances through which these inhibitory and accelerating influences are perceptible in metals vary with the conditions, the chief factors being the nature of the metals in contact and the electrical conductivity of the solution. An iron wire in contact with a metal of higher solution-tension (Al, Mg, Mn, Zn), and immersed in a 2 per cent. albumin-containing solution of K_3FeCy_6, remains free from filaments for a distance of several centimeters from the region of contact. Conversely, a strip of zinc in contact with a nobler metal (Pt, Cu, Fe) or carbon shows an accelerated formation of filaments for a similar distance. Theoretically, the current of the circuit extends through the whole of the metal and the adjoining solution; the intensity of the current between metal and solution at any point is a measure of the rate of electrolysis at that point (Faraday's law); this intensity is determined by the E.M.F. of the circuit and by the resistance of the intervening stretch of solution (that of the metal being negligible in comparison). The rate of action is thus greatest near the metallic junction, and grades off from there. A similar rule will hold for the formative or other influence of a circuit arising through local alteration in a living cell, tissue, or organism. Any local alteration of potential, due to injury, increased metabolic activity, or other condition, may thus exert an electrical influence at a distance from the site of alteration. In the case of a single cell, or other continuous element like a nerve, a current typically flows through the extracellular part of the circuit *toward* the region of alteration or physiological activity; presumably such a current produces at different points along its course the usual physiological effects of electrical currents, varying according to its intensity, local density, time-relations, etc. It is to be pre-

[1] *Cf.* Mathews, *Amer. Journ. Physiol.*, 1903, Vol. 8, p. 294.

[2] *E. g.*, the flow of a hormone-containing solution may depend on an electrical endosmose effect, etc.

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sumed that in a similar manner an actively growing region has a different potential from a quiescent region, and that the circuit which includes this active region influences processes in the tissue at a distance. Mathews found the regenerating end of a Tubularia stem *negative* relatively to other regions (in the same sense in which an active or injured region of, *e. g.*, a nerve fiber is negative; *i. e.*, within the living element the positive stream flows away from the active region); and he points out the possibility that the current thus traversing the tissue may influence metabolic and other physiological processes at different regions along its course, and thus may be an important factor in the initiation and coördination of the formative or other activities concerned in regeneration.[1] With this general conception I am in full agreement. The experiments described above show in how sensitive a manner the rate of formation of precipitation structures responds to variations in the intensity and direction of the local electrical currents traversing the system; and the structural and other conditions in living systems are sufficiently like those in the artificial models to warrant the belief that electrical currents must influence in a similar manner the physiological formative processes. The closeness of many of the above parallels between the behavior of the two classes of systems confirms this belief.

It should be emphasized that the mode of transmission which prevails in processes of growth and development appears to be fundamentally of the same nature as the mode of transmission of the excitation-state in irritable and conducting tissues like muscle and nerve. I have dealt with the special features of this latter mode of transmission in a recent series of papers in the *American Journal of Physiology*.[2] In this case the bioelectric circuit between the active and the inactive regions of the irritable element appears to be responsible for the transmission of the physiological effect; the current traversing the adjoining resting

[1] *Cf.* Mathews, *loc. cit.*, p. 299. The following quotation from Pfeffer's "Physiology of Plants" is also apposite here: "Weak electric currents continually circulate in plants"; these are "partly maintained by chemical and physical agencies at work in the plant itself. It is possible that these currents may influence metabolism," etc. (English translation, Vol. 2, p. 106.)

[2] *Amer. Journ. Physiol.*, 1914, Vol. 34, p. 414; 1915, Vol. 37, p. 348; 1916, Vol. 41, p. 126.

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region initiates there the same state of activity as that originally excited at the region of stimulation; the region which thus secondarily becomes active repeats the effect at regions beyond, and so the impulse spreads. There need be no decrement in this type of transmission, since an effect of the same kind and degree is automatically reduplicated at each point reached by the excitation-wave as it passes along the tissue.[1] In the transmission of the influence of a growing or regenerating or otherwise physiologically dominant region this reduplication is apparently not present, and hence a gradient of influence or "decrement" is shown. Otherwise the conditions are to be regarded as essentially the same in both types of transmission. It is possible, however, that combinations of the two may occur, giving rise to intermediate conditions.

Transmission of formative influence in development or growth, and transmission of the nervous type, are thus seen, according to the present theory, to be simply different manifestations of the same fundamental type of phenomenon, namely the transmission of an electrical influence associated with a flow of current around the bioelectric circuit. The physiological effects depend upon this current. Such a theory unifies a large number of apparently diverse phenomena. Thus the connection between growth processes and the assumed flow of current becomes intelligible if processes of electrolysis are in fact concerned in organic growth, as well as in the growth of the artificial formations described above. It also accounts for the rapidity of the nervous type of transmission in its most highly developed form, as pointed out in my earlier papers just cited. The transmission of the electrical influence through a circuit is instantaneous, whether

[1] Child assumes the existence of a decrement in the nervous type of transmission, similar to that observed in the transmission of formative influence; but this assumption seems to be unnecessary. The existence of trapped excitation-waves in rings of tissue, lasting in some cases for days—*e. g.*, in the medusa *Cassiopea* according to Mayer (*cf. Amer. Journ. Physiol.*, 1916, Vol. 39, p. 378)—proves that the impulse is renewed or recreated with undiminished intensity at each region reached by it in its course. In such a case there is obviously no decrement. It is now well established that the "all-or-none" law holds for nerve; this fact in itself would be incompatible with the existence of a decrement, since it implies that each region responds with the same degree of intensity, irrespective of the strength of the stimulus. The existence of a decrement in nerve during fatigue, anæsthesia, or asphyxia does not prove its existence under normal conditions.

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the conductor be electrolytic or metallic.[1] A local change of potential in a stretch of nerve thus produces instantly an electrical change at all points of the circuit constituted by the nerve and its medium, including therefore inactive regions of the tissue at a distance from the immediate site of alteration; and if the effect at any such region be sufficiently intense, a second state of excitation is there aroused, and a similar effect is transmitted from this second region to other resting regions beyond. As a striking concrete illustration of the instantaneous transmission of electrolytic effects along a conductor to a distance from the region immediately affected, I recommend the following simple experiment. A straight piece of copper or platinum wire, *e. g.*, 20 cm. long, is immersed in a vessel containing dilute H_2SO_4; the wire remains unaltered. If then one end is touched with a piece of zinc, instantly bubbles of hydrogen start out from the surface of the wire along its entire length. The transmission of the effects of a local stimulus in such a tissue as nerve is conditioned by a process of the same type, according to the present theory; hence it is independent of the transfer of material; and the velocity with which the impulse is propagated is limited only by the degree of sensitivity of the tissue to slight electrical disturbance and by the rapidity with which the local response takes place.[2]

[1] *I. e.*, the speed is that of transmission through the ether—equal to the velocity of light, or of electricity through a metallic conductor (apart from retardation due to electrostatic influences or self-induction). An experimental investigation of the velocity of the electric current through solutions is described in the book by Monroe Hopkins, "Experimental Electrochemistry" (London, 1905). The author reaches the conclusion: "An electrolyte solution conducts the electric current as rapidly as a conductor of the first class, regardless of its composition, provided we have an equal ohmic resistance of a non-inductive type" (p. 76).

[2] In a recent article (*Amer. Journ. Physiol.*, 1917, Vol. 42, p. 469) Mayer has objected to this theory of transmission (which I have called the "local action theory") on the ground that it is "too simple." Nothing, however, but the speed of transmission of the excitation-wave, and the identity of its effects with electrical effects (stimulation, inhibition, etc.), is directly explained by the theory in question. The *entire* phenomenon of nerve conduction is indeed exceedingly complex, including metabolic and other factors of a nature as yet imperfectly known; but the detailed nature of these factors need not be regarded by this general theory, which aims merely at accounting for the possibility and rate of nervous and other forms of physiological transmission where direct transfer of material does not enter. This it does by referring the phenomenon to a more general class, regarding which our

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Further light appears to be thrown upon the connection between bioelectric currents and formative processes by the above described phenomenon of rhythmical cilia-like vibratory movements of precipitation filaments. This phenomenon is frequent in newly formed filaments from iron and copper. The rhythm is typically slow, varying from one to five per second, and the movements are found only in the smaller filaments during the earliest stages of their formation, usually ceasing within a minute or two after their first appearance; at times, however, they may last for five minutes or more, especially with copper filaments. It appears probable that the phenomenon depends upon a rhythmical intermittency in the outflow of the solution contained within the tubular filament. As shown above, the metallic area at the base of the filament is an anode, the corresponding cathode being situated outside. The flow of solution through the tube is continuous so long as the extremity remains open, and is accompanied by an electric current. If, however, the opening be temporarily sealed by the deposition of precipitate, both the liquid flow and the electric current must at once cease; such a cessation of the liquid flow probably has a certain mechanical effect upon the position of the filament, while the electrical variation causes a change of surface-tension; and it seems likely that both effects combine to cause a movement, which has the effect of reopening the extremity of the filament and renewing the former condition. A rhythm is thus started which may continue for some time with often remarkable regularity. The determining condition of the phenomenon would then be the alternate disruption and reformation of a part of the tubular membrane, with corresponding opening and closing of the electrical circuit through the tube; the mechanical effects would result partly from the interruption of the liquid flow, but probably chiefly from the electrocapillary variation. Both the energy and the material for the process are furnished by the electric current through the tube.

knowledge is in many respects exact. If the transmission of the local excitation-state is due to the formation of local electrical circuits having the properties of electrical circuits in general, the difficulty of accounting for the rapid propagation of the excitation-wave along a highly irritable and rapidly responding element like a nerve-fibre at once disappears.

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The comparison with ciliary movement may with advantage be pursued somewhat further. In the living structure the continuance of the vibratory movement has been shown in many instances to be dependent upon the presence of the so-called basal particle (or "blepharoplast"), which is also the center out of which the structure grows in its development; severance of the ciliary filament from its base instantly arrests the movement.[1] A connection between the conditions of contractile activity and those of growth is thus shown; both processes depend upon changes taking place in the basal granule, which represents a specially modified portion of the protoplasmic surface layer, differing in chemical composition from other regions of the cell-surface (as shown by its staining reactions), and presumably also in electrical potential. This connection becomes intelligible if we assume that (just as in the above inorganic model) the basal particle represents an area from which a supply of formative and energy-yielding material flows out along the filament to replace the material which is altered or consumed at each ciliary stroke. This flow of material is to be regarded as associated with an electrical current, which being interrupted intermittently causes intermittent polarization-effects, with resultant variations of surface-tension producing the contractions. It is clear that a constant renewal of substance is necessary to maintain the integrity of the cilium and to furnish the energy of its contractions; and the resemblance between these structures and the artificial filaments described above appears to be too close not to be based upon some fundamental identity in the conditions of both their origin and their activity. The wide distribution of these vibratile cellular outgrowths, ranging from the lowest unicellular organisms to the highest animals, as well as the readiness with which they are formed at the free surfaces of cells, indicates that some general and simple condition determines their formation, and also that the conditions of their formation and of their contractile activity are closely connected.[2]

[1] *Cf.* Peter, *Anatomischer Anzeiger*, 1899, Vol. 15, p. 271; Verworn, *Pflüger's Archiv*, 1890, Vol. 48, p. 149.

[2] The fact that bioelectric variations in other active tissues (nerve, muscle) are so frequently rhythmical in character is significant in relation to the problem of the conditions of ciliary movement. It is possible that a rhythm or intermittency

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Another remarkable feature of these precipitation-tubules, already described, is that their formation is associated with a centrifugal flow of fluid along the tubule. This is not surprising when it is considered that they originate by a process of electrolysis at local electrical circuits. The passage of an electrical current through a porous diaphragm or other system of capillary tubes has long been known to involve a flow of the solution through the tubes; this is the phenomenon of fluid-transport by the current known as electrical endosmose. The possibility that this process plays a part in the transport of fluid through cells, *e. g.*, in secretion, has at times been touched upon by physiologists;[1] but in the absence of positive evidence it has been for the most part disregarded. Nevertheless it seems certain that currents of fluid must accompany the passage of electrical currents through or between cells, and also that the flow must be in general in the direction of the *positive* stream. Living protoplasm is a polyphasic system, largely fluid in its consistency, and pervaded by solid structural elements (membranes, fibrils, etc.) composed of colloidal material (protein, lipoid) which is charged negatively in contact with neutral or slightly alkaline media, such as those forming the tissue-fluids and the fluid part of protoplasm. The fluid layers in contact with such structures will accordingly be *positively* charged; and displacement of this fluid in the direction of the positive stream is inevitable if the protoplasm forms part of an electrical circuit. Protoplasm always contains salts and is a good conductor of electricity, and its enclosing membranes are readily permeable to water; hence the conditions for such a flow are present. Accordingly we should expect the passage of currents, bioelectric and other, through cells to be associated with a flow of fluid. Apart from such *a priori* considerations, there is ample evidence

in the state of electrical surface-polarization is general in living cells, and that the activity of cilia—contractile prolongations of the cell-surface—is one index of this condition. Any free cell-surface having intermittent polarization might thus give rise to cilia.

[1] *Cf.* Engelmann, *Pflüger's Archiv*, 1872, Vol. 6, p. 97; Waymouth Reid, *Philosophical Transactions*, 1900, Ser. B, Vol. 192, p. 239; Höber, *Pflüger's Archiv*, 1904, Vol. 101, p. 607. Experiments showing transport of water through tissues (muscle and nerve) by the constant current in the direction of the positive stream are described by Hermann, *Pflüger's Archiv*, 1897, Vol. 67, p. 240.

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in many physiological processes of a movement of fluid through cells, or from one region to another within cells, *e. g.*, the activities of gland-cells—which are normally associated with bioelectric currents flowing in the cells toward the secreting surface[1]—and the protoplasmic flowing movements in plant-cells. Certain recent observations of Chambers[2] on the micro-dissection of sea-urchin eggs illustrate the readiness with which flowing movements may be set up in protoplasm by local injury of the cell-surface, *i. e.*, by conditions which are known in general to render the cell-surface locally negative. The application of a needle to a sea-urchin egg in a hanging drop "produces peculiar currents in the egg-substance. The currents pass directly from the pushing object in a straight line through the egg to the anterior end where they curve outward and flow back along the surface to be caught again in the flow from the pushing object." The region of contact is presumably negative relatively to other regions of the cell-surface, *i. e.*, the positive stream flows outside the cell from the unaltered to the altered part of the surface and thence through the cell; the observed flow of fluid is thus in the direction of the positive stream. The possibility that dissolved materials may be thus transported from one region of the cell to another ought especially to be noted. If such a condition is general, a flow of fluid in reference to any temporarily altered region of the cell-surface will take place as a result of electrical transport; and this flow may largely determine the supply of material for the repair or reconstitution of the altered area. The fact that in the microscopic precipitation-tubules described in this paper a flow of fluid, due apparently to electrical transport, actually plays an essential part in the structure-forming process, is highly suggestive in relation to the problem of the conditions of structure-formation in cells. There is also evidence that a centripetal flow of fluid from the polar and extra-equatorial regions of the cell-surface takes place in eggs

[1] *Cf.* Langley, Schäfer's "Textbook of Physiology," Vol. 1, p. 517. Unfortunately this observation does not in itself indicate whether the electric current is the *cause* or *result* of the movement of fluid through the gland. A flow of solution through a porous partition, however caused, gives rise to an electric current.

[2] Chambers, "Microdissection Studies," *Amer. Journ. Physiol.*, 1917, Vol. 43, p. 1; *cf.* p. 7; also *Journal of Exper. Zoöl.*, 1917.

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and other cells during mitotic division, and that the astral rays are in part an expression of this flow, the central "sphere" representing an accumulation of the more fluid part of the protoplasm. Chambers brings evidence from micro-dissection favoring the view that the sphere is formed by the confluence of a system of radiating channels containing a more fluid "hyaloplasm" separated by radiating protoplasmic tracts of a solid or gel-like consistency. If the general surface of the dividing cell outside the equatorial area is electrically negative (in the usual physiological sense) relatively to this latter area, as the character of the form-change and certain other facts indicate,[1] then the positive stream in the cell-circuit must enter the surface at the polar and circumpolar areas and leave at the equatorial area. The conditions for a centripetal flow of fluid from the polar areas, due to electrical endosmose, would thus be furnished. The relation of such a flow to the general form-characters of the mitotic figure should be reconsidered in the light of these possibilities.

IV. The Possibility of Electrolysis at the Semipermeable Cell-boundaries.

Throughout the foregoing comparison between electrolytic precipitation-growths and organic growths it has been assumed that the formation of electrical circuits between different regions of the cell-surface, and associated with processes of the nature of electrolysis, is possible; and that the conditions under which such circuits arise are of the same essential nature as those determining the formation of local circuits between different parts of a metallic surface. This assumption may not appear at first sight to be consistent with our knowledge of the general conditions under which circuits are formed by chemical action, and electrolysis takes place. In the kind of process considered as typical by electrochemists, a part of the circuit always consists of a metallic conductor; this connects the two ion-forming or ion-combining surfaces (electrodes) which are in contact with the electrolyte-solution. In the living cell it is obvious that no conductor of the first class is present. In other respects, how-

[1] *Cf.* R. S. Lillie, *Journ. Exper. Zoöl.*, 1916, Vol. 21, p. 369.

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ever, the cell-surface resembles the surface of a metallic electrode; local alteration—injury, stimulation, chemical change—sets up local differences of potential, and currents flow between altered and unaltered regions. The potentials thus arising may be summed, polarizability is marked, and the cell-surface is water-insoluble—*i. e.*, there is a sharply defined boundary between protoplasm and surrounding medium. The chief difference in the conditions is that the conduction of electricity both inside and outside of the cell in a bioelectric circuit takes place entirely by means of conductors of the second class, *i. e.*, by ionic movement. Both protoplasm and medium are as a rule good electrolytic conductors; and the surface of separation consists of a thin semi-permeable partition composed of chemically alterable material which is of course non-metallic in character. Can true electrical circuits, in which chemical decompositions of the nature of electrolysis take place at the interfaces, originate under conditions of his kind? This question must be considered before the above comparison can be regarded as a valid one and acceptable conclusions be based upon it.

First of all it is necessary to guard against arbitrary preconceptions of the necessary character and arrangement of the different components present in an electrical circuit depending on chemical action, such as we are familiar with in the different types of battery. In all batteries there are at least two contiguous conducting media, the one metallic, the other consisting of an electrolyte-solution; at the boundary-surface between metal and electrolyte chemical changes takes place which furnish the current; the energy of the latter is thus transformed chemical energy. In the usual diagrammatic representation of these conditions, the terminals of the one conducting medium, the metal, are regarded as immersed in the other, *i. e.*, the electrolyte; these terminals, where electricity passes between metal and solution, are the *electrodes*, while the intervening stretch of metal is usually conceived as a wire passing through the air. This last, however, is obviously an arbitrary laboratory arrangement; it facilitates consideration of the processes at either electrode to have each isolated from the other, and the connecting wire removed from the possibility of chemical change; the regions where the essen-

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tial reactions occur are thus spatially distinct and the processes can be investigated separately. But evidently immersion of the conducting wire in the solution does not alter the conditions in any essential way. A piece of metal completely immersed in an electrolyte-solution, and of different composition or solution-tension at any two regions, or in contact at two areas with different solutions, is equally the seat of chemical change which is associated with the passage of an electric current between the two regions. This is the arrangement which corresponds more obviously to the conditions usually met with in nature. The case of local rusting in iron, or of the transmission of chemical influence (*e. g.*, excitation) from one region of a cell to another, exemplifies such an arrangement. It is important not to allow our conceptions of natural processes to be limited by the peculiarities of laboratory devices; these are always more or less arbitrary, and designed not only for convenience and reduplication at will, but with the special purpose of isolating the phenomena and making them as sharply defined as possible. In unmodified nature such conditions are rarely found; hence such resemblances as that between local chemical action in metals and the physiological effects following local alteration in living cells are not easily recognized.

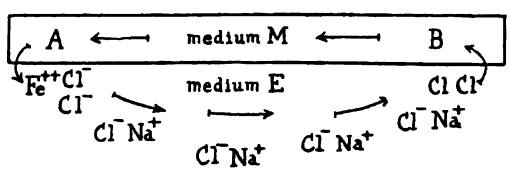

FIG. 1. In this diagram medium *M* represents the metallic part of the circuit, *e. g.*, a platinum wire, medium *E* the adjoining electrolyte solution, *e. g.*, NaCl. Ferrous chloride is in contact with the wire at one end (*A*), chlorine at the other (*B*). The arrows show the direction of the electrical current (positive stream) in the circuit.

A simple type of oxidation and reduction cell seems best adapted to illustrate the essential nature of the phenomena under consideration, especially since the energy of vital processes, including that of the bioelectric currents, is usually derived from oxidations. Probably the simplest case is that of an easily oxidizable salt, *e. g.*, ferrous chloride, in contact with a chemically

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indifferent conductor, *e. g.*, carbon or platinum, which is immersed in a solution of an electrolyte, *e. g.*, NaCl. The ferrous salt may be in contact with only one region of the platinum wire, *e. g.*, one of its extremities; if now an oxidizer (*e. g.*, chlorine, bromine, nitric acid, etc.) be brought in contact with the other end of the wire, the ferrous salt is at once oxidized to ferric. The subjoined diagram (Fig. 1) will illustrate, in which chlorine is regarded as the oxidizer. The rate of the process depends upon the concentrations of the $FeCl_2$ and Cl_2 at the electrode regions, the area of the electrodes, and the electrical conductivity of the circuit. Each atom of chlorine reaching the wire at region B causes instant oxidation of a molecule of $FeCl_2$ to $FeCl_3$; *i. e.*, each ferrous ion (Fe^{++}) becomes a ferric ion (Fe^{+++}) by receipt of a positive charge from the wire; this charge comes from the chlorine atom which releases it to the wire, becoming at the same time a chlorine ion (Cl^-). The number of chlorine ions in solution thus necessarily increases at the same rate as the number of ferric ions, and the only evident chemical changes are those occurring at the contact of the oxidizing and reducing substances with the wire. The current (positive stream) flows in the solution from the iron salt to the chlorine, and a certain process of rearrangement or change of ionic partners may be conceived as taking place throughout the whole intervening stretch of electrolyte-solution.[1]

The above account will indicate sufficiently the general character of the physical and chemical changes taking place in any oxidation and reduction cell. At the anodic area Fe^{++} ions are oxidized to Fe^{+++} ions; at the cathodic area electrically neutral chlorine atoms are reduced to Cl^- ions. The wire serves simply to convey electricity from the one region to the other. Similarly

[1] It is impossible to give a full exposition in this place, and the reader is referred to the textbooks of electrochemistry (Arrhenius, LeBlanc, Lüpke, etc.) for a more detailed treatment. The electron theory includes all of the above phenomena as special cases. From the standpoint of this theory it is more correct to regard each Fe^{++} ion as yielding an electron (negative charge) to the wire at region *A*; this displaces a corresponding electron at *B*; this electron is taken up by the Cl atom which thus becomes a Cl^- ion. The flow of electrons constitutes the current in the wire; at the electrodes there is equal and simultaneous loss and gain of electrons by the substances (ions or other dissolved substances) at anode and cathode respectively.

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any oxidizer which is capable of being reduced by $FeCl_2$ may be substituted for chlorine; and any substance which is capable of being oxidized by the oxidizer may be substituted for the ferrous salt.[1] The above substances serve merely to furnish the simplest possible illustration. Oxidation at the anode, reduction at the cathode, with accompanying flow of current between the two regions, form the constant features of the phenomenon; obviously the energy of the current is derived from the energy of the oxidation. It will thus be readily understood that the chemical changes at the electrodes may in some cases be much more complex than those cited above for illustration; and that under certain conditions they may result in decompositions and syntheses of a far-reaching character.

In the above diagram one half of the circuit, designated as medium *M*, is regarded as consisting of a metallic conductor which itself undergoes no chemical change; the other half, medium *E*, is the electrolyte solution. Obviously a metallic conductor which is not chemically indifferent but is capable of combining chemically with the ions of the electrolyte-solution will produce similar electrical effects. A metal of low solution-tension like zinc or iron will contribute cations to the solution at the anode; these will form chemical combinations according to the nature of the substances present in the solution. Or the reducing influence at the cathode will affect all reducible substances there present; if, *e. g.*, the cathode is composed of a conducting oxide like copper oxide, as in the Edison cell, this is reduced to the metallic state. Evidently the more complex the material of anode and cathode, and the more complex the contiguous solution, the more numerous the chemical transformations that may occur. In all cases, however, Faraday's law holds, and the energy freed in the resulting process is strictly dependent upon the difference in the chemical potential of the substances undergoing transformation at the two regions.

The case does not appear to be altered in essential principle when medium *M* is considered to be not a metal, but a second

[1] See the text-books of electrochemistry for examples of the various possible combinations. An oxidizable organic substance, *e. g.*, pyrogallol, α-naphthol, sugar, may be the substance at the anode; a peroxide the oxidizer at the cathode. These examples are adduced to suggest biological possibilities.

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electrolyte-solution, separated from medium E by a thin partition which prevents diffusion (*i. e.*, is semi-permeable) and can undergo the chemical changes necessary for the generation of an electric current, *e. g.*, oxidation at one region simultaneously with reduction at another. Under these conditions medium M may be permanently of a different composition from medium E, both are electrical conductors of the electrolytic kind, and local chemical changes resulting in ionization or deionization within the substance of the partition-film will result in corresponding electrical effects which may be transmitted and produce chemical effects at a distance just as in the case considered above. This appears to be the type of arrangement exemplified in the living cell.

To understand how such currents may be formed without a metallic conductor it is necessary to consider more closely the nature of the part played by this conductor in the usual type of circuit. In the above example the platinum wire serves simply as a means for transmitting electricity, a positive charge passing to (or a negative away from) the metal from each Cl atom and from the metal to each Fe^{++} ion. Electroneutrality is thus preserved on either side of the interface; the only region where chemical change occurs is where electricity is thus added to or abstracted from the substances in contact with the electrodes. This last is the essential condition of all electrolyses; obviously the precise character of the chemical transformations in any special instance depends on the nature of the substances present at the electrodes. Extensive molecular rearrangements may take place under some conditions; thus the anodic oxidation may result in syntheses as well as in decompositions; similarly with the reductions at the cathode. In the interior of the wire no permanent change occurs, since at each region electrons are simultaneously entering from the one side and leaving from the other in equal quantity, leaving the local condition unaltered. In the interior of the solution diffusion-changes may occur; but apparently there are no interionic or intermolecular transfers of electrons, since the quantity of electricity passing through the circuit is strictly proportional to the number of ions reaching the electrodes and charging or discharging there (Faraday's

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law).[1] The source of the current is thus the chemical changes taking place at the electrodes; in these changes electricity is added to the wire at one region and an equal quantity is simultaneously abstracted at another. Precisely the same chemical change takes place and the same quantity of energy is freed when the chlorine is brought directly into contact with the solution of ferrous salt; here the transfer of negative electricity from the ferrous ions to the chlorine atoms takes place without the intermediary of a metallic conductor. These considerations show that the metallic conductor, by acting as a source of electrons, merely *enables* the change of valence to take place without the direct contact of the chlorine and the ferrous salt. This effect follows because the affinity of the chlorine atom for a negative charge (*i. e.*, electron) is greater than that of the ferrous ion; the chlorine atom is thus able to abstract a negative charge from the metal and impel the ferrous ion to yield up a similar charge to it,—*i. e.*, to assume an additional positive charge and become a ferric ion. The latter then interacts chemically with whatever other substances are available.[2]

In the oxidation and reduction circuit considered above, the current on the one side of the interface, *i. e.*, in the metal, is conducted by a simple movement of electrons without chemical change; on the other side, *i. e.*, in the solution, it is conducted by the diffusion of ions and the charging or discharging of ions at the electrode-areas, with consequent local chemical change. Let us now suppose that instead of the combination of a metallic conductor and an electrolyte-solution, we have *two* electrolyte solutions separated by a thin semi-permeable partition the substance of which can combine chemically with the ions of one of the solutions. Such an arrangement corresponds to the case

[1] Were this otherwise, electricity could flow through the solution by shifting of electrons from ion to ion (or molecule to molecule, etc.) without the transport of the ion as a whole. This, however, does not seem to occur (unless the current-intensity is very high). It is only when the ion interacts chemically with the substances at the electrode, or with the electrode itself, that any flow of current can take place. This is why electrolysis is always associated with the passage of a current through a solution. The metallic state appears to be characterized by a relatively high concentration and free mobility of electrons.

[2] The presence of potassium thiocyanate in the solution at the anode is a convenient way of demonstrating the production of ferric ions. The ferrous salt should be as free as possible from ferric admixture.

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of the living cell immersed in its normal medium. The two solutions are to be conceived as differing in their electrolyte-content, so that a potential-difference exists across the membrane. If now we consider a circuit as arising between different regions of the surface in consequence of some local chemical change in the substance of the partition, such as an oxidation, it is clear that the conditions of the flow of current in both solutions must be in principle similar to those just considered; *i. e.*, the mode of current-transport accompanied by chemical change which prevails on one side of the interface in the example cited, *i. e.*, in medium E, may equally well prevail on *both* sides. The essential conditions are that the chemical changes taking place at the interface must be of such a kind as to furnish free energy (for the flow of current), and that the necessary electro-neutrality of either solution is preserved intact. It is not difficult to imagine the general features of such an arrangement, although as regards the special nature of the conditions existing in living cells (the substances involved, the nature of the reactions, etc.) little definite can be said at present. In brief, then, the present conception regards the current in the interior of either solution as transported by the movement of ions in the usual manner; but in its transport across the boundary-partition chemical reactions are concerned; *i. e.*, in this part of the circuit the essential change consists in transfers of electrons between interacting molecules or ions with corresponding chemical reactions which furnish the energy of the current. Such processes would be conditioned in the same essential manner as the processes at metallic electrodes, *i. e.*, would be of the nature of electrolysis, since they would depend upon the flow of the current through the circuit. Hence they would be influenced by electric currents from outside sources, besides giving rise to currents when they occur of their own accord under normal conditions. It seems highly probable that the characteristic electrical properties of living cells depend upon arrangements of essentially this kind; the fundamental general fact that cell-activities both give rise to electrical currents, and are themselves profoundly influenced by currents of outside origin, is most readily understood on this hypothesis.

The special nature of the conditions in living cells may now

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be briefly considered, although for the present little more can be done than to indicate the probable class to which the phenomena belong; the elucidation of details is a task for future research. In general the film separating the protoplasm from the external medium is thin and semipermeable, and the solutions on either side of this partition are of different composition. Hence the conditions for a potential-difference between protoplasm and medium exist; and such a state of electrical surface-polarization is always found. This polarization varies characteristically with the nature and the physiological activity of the cell; and circuits are thus continually arising in active tissues (like muscle, nerve, etc.) between different regions of the cell-surface. The conditions under which the normal bioelectric circuits originate seem most closely analogous to those prevailing in such an arrangement as the "oxygen-hydrogen cell" of electrochemistry, in which two platinum electrodes, charged respectively with oxygen and hydrogen, are immersed in an electrolyte-solution. In this case the oxygen electrode forms the cathode, the hydrogen, the anode; the oxygen gives up positive charges to the platinum when the circuit is closed, while the hydrogen receives such charges from the metal; the oxygen thus forms oxygen- or hydroxyl-ions, the hydrogen forms hydrogen-ions; and the union of these to form water furnishes the energy of the current. Let us suppose—to make the case concrete—that the external solution bathing the living cell contains oxygen, and that the surface-film consists of oxidizable material. In the resting condition of the cell there is a certain tendency for the oxidation to take place (or oxidation-tension), *i. e.*, for the oxygen to impart positive charges to the oxidizable partition, becoming itself ionized; but this tendency is held in check—just as in the oxygen-electrode when the circuit is open—because such a transfer would leave unbalanced negative charges behind in the medium. Hence an equilibrium exists, similar to that at the surface of any battery-plate with open circuit. But any change of condition by which negative charges may be set free in the protoplasm at any region will disturb this equilibrium and allow the combination to take place. The oxygen then imparts its positive charges to the oxidizable material of the partition; this

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on becoming positive may then combine with the negative ions present—which may be oxygen or hydroxyl or other available anions, according to concentrations, affinities, etc.—and the material assumes a higher state of oxidation; other transformations, including syntheses, may follow from this change. Such a process implies that a positive electrical stream enters the cell-surface from the medium at the site of oxidation, and leaves the cell at other regions; at the latter regions presumably cations are given off from the cell-surface (which appears to act as a reversible electrode relatively to cations). A circuit thus flows through cell and medium, the positive stream flowing in the external medium toward the region of oxidation and away from the resting or chemically inactive regions. In other words, the local combination of oxygen with the cell-surface at any region involves the entrance of positive charges at that region, in a manner analogous to the entrance of positive charges at the cathode in the oxygen-hydrogen cell. So far as the physiological evidence extends, the region of highest oxidation-rate in cells does appear always to be negative (in the physiological sense) toward regions of lower oxidation-rate. This is indicated by the general fact that stimulated or active regions are negative relatively to inactive regions, oxygen being typically required for such activity; actively growing regions appear also to be negative relatively to less active or non-growing regions; this is seen in the regeneration of hydroid stems and of the tails of fishes, and in the growth of seedlings,[1] and is probably general, although at present the observations are insufficient to establish this generalization empirically. In such cases the negative regions—*i. e.*, the regions where the positive stream enters the cells from outside—are the regions of most active constructive metabolism; and since oxygen is required for growth and regeneration, this fact may be regarded as an expression of the general connection between oxidation-processes and syntheses. It should be added that changes in the local physical or chemical

[1] *Cf.* Mathews, *loc. cit.* For the case of seedlings *cf.* Müller-Hettlingen, *Pflüger's Archiv*, 1883, Vol. 31, p. 193. Hermann first observed that the growing root-tip in seedlings was strongly negative to other regions (*Pflüger's Archiv*, 1882, Vol. 27, p. 288), and Müller-Hettlingen followed the subject further at Hermann's suggestion.

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condition of the surface-film, induced (*e. g.*) by external agents, may also produce bioelectric circuits accompanied by oxidation processes; thus a region of increased permeability resulting from stimulation may be the condition disturbing the equilibrium; or a local metabolic change may take place in the surface film resulting in abstraction of cations from the cell-interior by a process of reduction; in a circuit like that imagined above such a change would result in oxidation at other regions of the cell-surface. The conditions under which local oxidations occur and bioelectric circuits originate undoubtedly vary widely in different cells, and probably also in the same cell at different times.

It thus seems clear that electrical circuits may originate through chemical reactions which take place at the boundary between solutions of different composition separated from each other by semi-permeable partitions having the properties of the plasma-membranes of living cells. There is no theoretical necessity for a conductor of the first class. Local transfers of electricity to and from the protoplasm may occur simultaneously at two different regions of the cell-surface as the result of chemical interaction between the substance of the surface-film and ions or ion-yielding substances present in the protoplasm or in the medium; circuits between these regions thus arise. The possibility of this general condition must be admitted if we accept the general electrochemical theory that the metallic conductor in (*e. g.*) an oxidation-reduction cell enables the reaction to take place simply by serving as medium for the transmission of electrical charges. The phenomenon of chemical action at a distance appears indeed to be a clear demonstration that chemical action depends upon the transfer of such charges. The production of the bioelectric circuits becomes intelligible if we assume that the charges conditioning the reactions at the electromotor surfaces may be derived from oxidizing or reducing substances present in the protoplasm, or surroundings, just as readily as from a metallic conductor.

This general conception is evidently related to the conception of ionic exchange in such a phenomenon as absorption.[1] Local

[1] See Höber's "Physikalische Chemie der Zelle," pp. 238 seq.

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circuits should be equally possible in such cases, and these may be associated under certain conditions with chemical change. The action of strong electrolytes upon poorly conducting material (*e. g.*, wood, rubber, leather, etc.) is probably accompanied by electrolytic action of this or similar kind. The distinction between ordinary chemical action and chemical action due to electrolysis is in fact rendered arbitrary by the facts of chemical action at a distance. Wherever electrical transfer is possible under conditions not resulting in the development of a counter E.M.F. or other polarizing effect which arrests the current, the chemical influence may be transmitted to a distance. This explains why circuits are necessary to this process. Of course the rate of any reaction thus conditioned varies inversely with the electrical resistance of the circuit which determines it; and it is only when the resistance is relatively low—as in circuits consisting of a metallic conductor combined with an electrolyte-solution, or in circuits of two electrolyte-solutions separated by a polarizable semi-permeable partition—that such transmission can take place through considerable distances.

A difficulty may be felt in accounting for the conduction of electricity across the membrane in living cells. This is normally *semi-permeable*, *i. e.*, impermeable to ions and other diffusing substances; the continued existence of the cell depends in fact upon this condition. The assumption of a selective permeability to cations (or reversibility to cations) may remove the difficulty of explaining how the current can be conducted across the resting regions of the cell-surface. At the active regions conduction may take place by chemical reaction accompanied by electron-transfer, as already suggested. It seems possible, however, that at times the positive stream may pass from the cell-interior to the external medium without such chemical change; thus the addition of cations to the membrane from within, following stimulation at some other region, might disturb the polarization-equilibrium and detach corresponding cations from the outer surface; at such regions the current would flow from within outward without change in the membrane, as observed at the resting regions of the cell. In such a case the membrane would behave like a non-polarizable electrode. To what degree and

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under what circumstances this possibility is realized is difficult to say. Certainly in most of the instances hitherto investigated (muscle, nerve) the passage of the constant current through the cell produces marked polarization-effects (*i. e.*, sets up immediately a strong counter E.M.F.). Nevertheless the above possibility should be considered.

V. General Conclusions.

The foregoing discussion raises the question whether in organic growth the essential structural condition is not the presence of semi-permeable and hence electrically polarized partitions separating the living substance from its medium, and at which processes of electrolysis may take place. If this is so, the prevalence of the cellular type of organization would be largely accounted for. Organic growth (as well as normal maintenance) would then involve the deposition in the continuous surface-layer of materials which preserve the character of that layer, *i. e.*, its continuity, semi-permeability, and specific physico-chemical composition. It is clear that any layer having such properties would have to form a *closed surface;* otherwise the difference between the inner and outer solutions could not persist; hence a "cell"—mass of protoplasm enclosed by a membrane—would be the living unit. A tendency to a continual increase of this surface-layer in an appropriate nutrient medium would then constitute the primary feature of the growth-process. Evidently the continued growth of such a system would involve a decrease in the ratio of surface to volume: and the need of preserving this ratio above a certain minimum may be the condition that has led to the association of cell-division with growth, as Herbert Spencer suggested long ago. According to this conception, "living" material would be essentially material at the boundary-surface or under the direct influence of the boundary-surface; definite limits would thus be imposed upon the dimensions of the individual living units. In general we find that protoplasm is partitioned freely; *i. e.*, the ratio of surface-protoplasm (membrane-protoplasm) to the total mass is usually large. The exceptions (yolk-laden eggs, muscle cells, certain plant-cells) may be explained on special grounds.

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There is no doubt that the distinctive vital properties of self-maintenance, growth, and reproduction are closely interconnected; it would probably be more correct to say that they are manifestations of the same fundamental physiological activity under somewhat different conditions. The essential feature of this activity is constructive metabolism of a specific kind. The mechanism determining this process of construction must be intimately connected with the mechanism determining the response to stimulation, since stimulation, which effects a breakdown of the living substance, must itself form the condition of the restitution of the broken-down material, if the system is to continue to exist. This was clearly recognized by Claude Bernard.[1] Some kind of self-regulating cycle, in which a destructive process calls forth automatically or inevitably a counter-process of repair, would thus seem to be the necessary foundation of any form of vitality. An analogy to this condition is seen in the chemical processes of any electrolytic circuit; just as oxidation at the one electrode involves the inverse process at the other electrode, so decomposition or structural breakdown at one region of the cell-surface may, by giving rise to a local bioelectric circuit, directly determine a reparative synthesis either at the same or at an adjoining region.[2] Some general physico-chemical condition of a simple kind must be common to *all* of the various types of living system, since they are all *living*. The universality of the cellular type of organization seems—to my mind at least—to indicate that the essential structural condition in living matter is the presence of polarizable partitions at which processes of electrolysis, including both syntheses and decompositions, are to be conceived as taking place under the influence of local electrical circuits. The self-maintaining character of the living system, its responsiveness to outside influence (especially of an electrical kind), and the transmission of chemical influence to a distance, become to a considerable degree intelligible on such an hypothesis. These considerations may appear too speculative to many; but further advance in physiological analysis seems to demand that the fundamental vital processes should at least be

[1] *Cf. e. g.*, "Leçons sur les phénomènes de la vie," Vol. 1, p. 127.

[2] For further discussion of this possibility *cf.* my recent paper, *Amer. Journ. Physiol.*, 1917, Vol. 43, pp. 56, 57.

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assigned to their proper physico-chemical classes. The similarities between the phenomena described in this paper and many of the most characteristic peculiarities of the growth-process in organisms seem to me to be too detailed not to signify an identity in some essential underlying condition. The need of a physico-chemical analysis of the growth-process is clear; growth is unquestionably the most fundamental of vital activities, and any adequate theory of growth would be equivalent to a theory of the vital process itself.

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THE CONCRESCENCE OF FOLLICLES IN THE HYPOTYPICAL OVARY.[1]

LEO LOEB.

In a preceding paper we have shown that lack of sufficient nourishment is the cause of a hypotypical condition of the ovaries. In one guinea pig we found in addition to the hypotypical condition another peculiarity of the ovaries, which is of considerable interest and which deserves a special description. In connection with the facts reported in the preceding paper it leads to some interesting conclusions as to the effect of underfeeding on the development of the ovarian stroma, and on the development of what in the guinea pig in certain respects corresponds to the interstitial gland of the rabbit and it may throw light on the origin of follicles containing more than one egg.

Female guinea pig No. 383 was obtained from Iowa. On January 12, 1917, it weighed 475 grams; on that date both lobes of the thyroid of this animal were removed. One thyroid lobe from another guinea pig, obtained from a different breeder and weighing 380 grams, was transplanted into the subcutaneous tissue of guinea pig No. 383. The transplanted thyroid was removed for microscopic study seventeen days later, January 29. At that time the guinea pig weighed 322 grams; it had therefore lost 32 per cent. of its original weight in a period of seventeen days. The transplanted thyroid was on the whole in a very good condition; we shall refer to this aspect of our findings in another connection. Here we are especially concerned with the condition of the sexual organs. The uterus shows low or medium cylindrical epithelium of the surface and glands with a very small number of mitoses in the surface epithelium. The mucosa is thin and fibrillar but very hyperemic. In the lumen of the uterus there are some erythrocytes. The ovaries are markedly hypotypical. They contain several atretic yellow bodies, the rem-

[1] From the Department of Comparative Pathology, Washington University, Medical School, St. Louis.

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nants of former corpora lutea. There are in the cortex a number of primordial follicles developing into small Graafian follicles and into follicles of small to medium size. But before they have hardly reached the latter size, a slow solution of the granulosa sets in and very soon the cavity of the follicles is lined by the theca interna.

The further course of the atresia of the follicles is, however, changed; the connective tissue does not as usual grow into the follicular cavity; instead the atretic follicles present themselves as small cysts. The ordinary follicles in various stages of connective tissue atresia are therefore lacking. There is, however, present a very large number of follicles in the last stage of atresia, a cavity with remnants of the zona pellucida, or merely a small cavity surrounded by a thick layer of the shrunken theca interna cells. By far the greater part of the ovaries consists of such follicles in the last stage of atresia; the proportion of such follicles is much greater in this animal than in normal ovaries which are in the period following the first week of ovulation and even larger than in the other hypotypical ovaries which we observed.

There are two further peculiarities noticeable in these ovaries, viz., (1) a relative decrease in the amount of fibrous tissue which surrounds the various follicles, and (2) the presence of numerous follicles with multiple eggs. While we observe around a number of growing or atretic follicles a fibrous membrane, the latter is always thin and it is absent around other follicles. Not rarely we see two adjoining follicles of small to medium size merely separated by several rows of relatively large theca interna cells; or we see small follicles close together, and not separated by fibrous tissue; at other places the theca internae of two quite atretic follicles press on each other, the theca cells of the one follicle surrounding directly the capillaries of the theca interna of the adjoining follicle. In other cases two or three shrunken egg cavities are enclosed in one mass of small theca interna cells.

If we compare the amount of fibrous tissue separating the follicles in this animal and in other animals with either normal or hypotypical ovaries, we find in the ovaries of other guinea pigs a larger amount of fibrous stroma separating the various follicles. This decrease in fibrous tissue made it possible to

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differentiate microscopically the ovaries of this animal from those of other guinea pigs. There occurs in these ovaries a considerable number of follicles with two, three or even more eggs. We see in the most peripheral zone of the ovary two well preserved eggs of about the same size, but one slightly pressing upon the other and both surrounded by a single layer of granulosa cells. Somewhat further away from the tunica albuginea we see small follicles with two eggs of the same size, separated only imperfectly by a few flat granulosa cells. At other places we see follicles of small to medium size with a cavity. At the end of the cavity directed toward the center of the ovary there is a relatively large, well-preserved egg, surrounded by several rows of granulosa cells. At the side of the follicle directed toward the periphery of the ovary there is a very small, but well-preserved egg, in an earlier stage of development, surrounded by only one layer of granulosa cells. Here we have a combination of a primordial and of a small Graafian follicle. In other cases we see a small egg of a primordial follicle, pushing its way through the granulosa of the somewhat larger follicle and just reaching the cavity with the outer pole; the most frequent orientation of the two eggs is as follows: The larger one, surrounded by several rows of granulosa cells, is placed at the central pole of the cavity and the smaller one more toward the peripheral pole. But occasionally the order may be reversed, or the small egg may be at the lateral aspect of the follicle. In one case we found the egg of the primordial follicle in the suspensory ligament of the granulosa of the larger follicle. In still other cases we find a combination of a small egg at an early stage of its development, surrounded by one row of granulosa cells, the whole corresponding to a primordial follicle in the cavity of a small to medium follicle in which the granulosa and egg have already undergone degenerative changes. The degenerating ovum may still be surrounded by granulosa cells or the granulosa cells may have been dissolved. In other cases we find in a follicle of small to medium size two well developed eggs, both surrounded by several layers of granulosa cells. Similar combinations we find in follicles with three eggs. There may be combined a degenerated egg, a relatively large, preserved egg surrounded by several layers of granu-

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losa, and an egg of a primordial follicle enveloped by one layer of granulosa. In a follicle in which we found a still larger number of eggs, the latter were well preserved and rather small, and situated in different parts of the granulosa. Of interest is also an observation which showed that a disintegrating egg may enter into direct contact with the theca interna of a nearby follicle of small to medium size.

These are the principal combinations of double or multiple eggs we observed in the ovaries of this animal, and both ovaries behaved in a similar manner. How shall we interpret this condition? The large number of follicles with two or more eggs which we found in both ovaries indicates clearly that we have to deal with a general condition of the organism affecting both ovaries equally and not with a local ovarian change. We may conclude that bioval or plurioval follicles may originate in either of the two following ways: (1) The young eggs in the tunica albuginea of the ovary remain united; connective tissue fails to grow between two or more eggs and to separate from each other the different eggs with the surrounding granulosa cells. The pictures of primordial follicles containing two eggs speak in favor of this interpretation. (2) But other follicles containing more than one egg must originate in a somewhat different manner. Very small follicles, especially primordial follicles, push their way into larger follicles, perhaps even into such follicles which are already in the process of degeneration. And this is probably by far the more frequent mode of origin. We find different stages in the junction of two follicles of different sizes, and in different stages of development. We can follow the pushing in of the smaller into the larger follicles. The fact that the thickness of the granulosa surrounding the different eggs in a follicle is in accordance with the character of the egg is only compatible with the view that two formerly distinct follicles effected a secondary union. In favor of this interpretation speaks also the observation that in the majority of cases the more undeveloped egg is situated toward the outer pole of the larger follicle. This is in accordance with the fact that the smaller a follicle is, the more peripheral is its situation. Occasionally, however, a primordial follicle may be pushed deeper into the cortical tissue

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before it enters the wall of the larger follicle. In this case we might expect to find the smaller egg in the central part of the follicle and the larger one more toward the periphery. Whether also two small to medium follicles of similar size can unite to form one follicle with two eggs, we are unable to determine with certainty.

Essentially both modes of origin are in all probability based on the same condition, both being due to the same cause, namely, the relative inactivity of the connective tissue in these ovaries. We saw that in this animal the atresia of the follicles does not take its normal course, but that after disintegration of the follicles the connective tissue fails to grow into the cavity and that thus small follicular cysts syrrounded by theca interna are formed. We saw furthermore that the fibrous bands separating the various follicles are not so well developed as in the ovaries of other guinea pigs. It is therefore very probable that this deficiency in the connective tissue is at the bottom of this condition. The relative inactivity of the connective tissue, its failure to proliferate and to produce fibrous tissue, accounts for the imperfect atresia of the follicles and the imperfect separation of the follicles, and it is this inactivity of the connective tissue which is responsible for the occurrence of the follicles containing multiple eggs. In the first place, the connective tissue in some cases fails to grow properly between the very young eggs and their granulosa and to separate the two follicles; thus the first mode in the production of follicles with several eggs is brought about. It is due to a pathological persistence of an earlier stage in the development of follicles. And secondly the lack of development of strong fibrous tissue around a number of follicles, together with the pressure existing in the ovary as the result of the growth of young follicles, is responsible for the pushing of small follicles into larger ones. Thus there is no essential difference in the mode of origin of bioval or plurioval follicles at various stages of development.

The next question concerns the cause of this relative inactivity of the connective tissue. The study of the hypotypical follicles, which we reported in the preceding paper, throws light on this problem. We mentioned there that in cases in which the hypo-

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typical condition was pronounced, there was noticeable a tendency to delay in the ingrowth of connective tissue into the follicular cavity; thus small cysts resulted. We interpreted this relative inactivity as due to the underfeeding which at last affects also the connective tissue. This condition represents a further stage in the chain of changes produced through underfeeding. This chain is as follows: (1) The maturation of follicles is suspended. (2) Abortion takes place in pregnant guinea pigs. (3) The granulosa cells of developing follicles are injured and are prematurely dissolved, but the relative strength of new formation on the one hand, and of destruction of granulosa cells on the other hand, shows such a balance in favor of new production that the development of follicles progresses so far that medium follicles are formed. (4) This balance becomes more unfavorable and only small and small to medium follicles develop; after this stage has been reached and the granulosa has been dissolved connective tissue grows in and fills the cavity and leads to a shrinking of the follicle. (5) In the last stage even the connective tissue becomes inactive. The ingrowth of connective tissue into the cavity does not take place. And the same process leads also in all probablity to an under-development of the fibrous bands separating the various follicles. Thus the separation of follicles becomes interfered with and the multiplicity of eggs in a follicle results.

It is, of course, possible that there are at work additional factors which favor such a process. It might be that in certain individuals the tendency of connective tissue to be inactive is greater than in others. It might also be that the number of the young follicles in early stages of development is greater in this animal and that thus a crowding of young follicles results. However, if such a difference exists between this and other ovaries, it is not very marked. Multiplicity of eggs in the ovarian follicles has been observed by us in several other ovaries of guinea pigs. At the time however when our previous observations were made we had not yet gained an undestanding of the connection between this change and the abnormal conditions which we found in this case. Ovarian follicles with several eggs occur also in other species, especially also in man. It would be

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of interest to inquire whether in all cases the causes are the same. or whether different conditions may lead to the same result; In such an inquiry we have to keep in mind the possibility that a hypotypical condition of the ovaries might be a transitory state; that after having led to the production of follicles with multiple eggs, new follicles may again begin to develop normally, so that in the end nothing indicates that at one time such ovaries had been hypotypical.

This concrescence of follicles is somewhat analogous to a condition we find in other glands. In the thyroid of the guinea pig, as well as in the mammary gland of the mouse, we found occasionally structures which could only be explained as due to a concrescence of neighboring acini; due to the disappearance of the walls separating adjoining acini. We have therefore to deal with a phenomenon of a more general character, which under certain conditions may perhaps occur in all the glands.

There was in the ovaries of this guinea pig an additional feature which deserves special mention, viz., the very marked development of what corresponds to the interstitial gland in the rabbit. As we stated above, by far the greater part of this ovary consists of follicles in the last stage of atresia, follicles in which the shrunken cells of a well-developed theca interna surround one or several small cavities, the remnants of what were formerly follicular cavities. While we find in all hypotypical ovaries a relative preponderance of this kind of follicle, in the ovaries of this guinea pig this feature was more prominent than in the ovaries of the other animals. This is due to the following factors: (1) At the time of the thyroidectomy, the ovaries were in all probability relatively large, the animal weighing at that time 475 grams; thus space was available for the expansion of atretic follicles. (2) The follicles ceased to develop to medium or large size. Thus the pressure exerted by the larger follicles was eliminated, and a chance was given the atretic follicles to occupy the space otherwise occupied by large follicles. The slight development of fibrous bands around the follicles must have a similar effect diminishing pressure exerted on atretic follicles. We may therefore conclude that the structure analogous to the interstitial gland is prominent whenever the intra-

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ovarian pressure is relatively slight. Under these circumstances the last stage of follicular atresia is preserved throughout a relatively long period of time. A similar condition we find in the ovaries of the guinea pig within the first week after ovulation, when the larger follicles have all become atretic.

Conversely we may conclude that the intraovarian pressure hastens the disappearance of the theca interna and of the other vestiges of atretic follicles. The pressure of the growing structures is stronger than the pressure of the resting and disappearing structures, and thus the life and growth in one part of the ovary hastens the disappearance of degenerating structures elsewhere. Not only is the disappearance of the shrunken theca internæ thus hastened, but also that of the fibrous tissue structures in the ovary. In our case it is uncertain whether the connective tissue had formerly grown into the follicles which are now in the last stage of atresia, this connective tissue having been absorbed subsequently, or whether in this animal the last stage of connective tissue atresia was reached directly from the cystic condition through absorption of the fluid in the follicular cavity, without a preceding ingrowth of fibrous tissue. In case the latter alternative should hold good the lack of ingrowth of connective tissue into atretic follicles would have been present for some time previous to the examination of the ovaries. In some atretic follicles we notice however some vestiges of fibrous tissue around the central cavity and it is therefore probable that in these cases the connective tissue had previously grown into the cavity after the solution of the granulosa. We also must consider the possibility that diminished intraovarian pressure may in itself influence the activity of the connective tissue.

Summary.

We describe the ovaries of a guinea pig in which the experimentally produced hypotypical condition was more advanced than in any of the other animals which we observed. In this case the underfeeding not only affected the activity of the granulosa, but also of the connective tissue. In consequence of a lowered activity of the connective tissue a concrescence of follicles takes place in numerous cases in both ovaries. A lowering of

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intraovarian pressure in such ovaries leads to a relative preponderance of what corresponds to the interstitial gland in the ovaries of certain other species. The concrescence of ovarian follicles is analogous to the union of neighboring acini in the mammary gland and thyroid. It is a phenomenon which occurs probably in all or the majority of glandular structures.

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A COMPARISON OF MITOCHONDRIA IN PLANT AND ANIMAL CELLS.[1]

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The recent discovery, through the elaboration of new methods of technique, of the wide distribution of mitochondria has attracted the attention of many investigators in widely separate fields. Their characteristic form, strongly suggestive of bacteria, is now quite familiar. The technique was devised by anatomists and was applied by them to plant cells. I refer particularly to Meves' ('04, p. 284) work on *Nymphæa*. But, as might have been expected, these structures did not altogether escape the careful scrutiny of the older botanists, despite their imperfect methods of technique; for Zimmermann ('93, p. 215) certainly observed and described mitochondria in the living hair cells of *Momordica elaterium* and in the meristem and root tip of *Vicia faba*. Nevertheless botanists in this country have been slow to study mitochondria notwithstanding the fact that in properly made preparations they constitute a cell organ as conspicuous as the nucleus.

Unhappily we have deplorably little experimental evidence to lead us to any conclusion as to their functional significance in the cell economy. Our curiosity has been so insistent that we have resorted to less reliable sources of information, one of which is the argument from analogy. It is said that since the mitochondria are found in almost all active cells, their function must be a generalized one which they all possess in common. The validity of this line of reasoning rests entirely upon the resemblance of mitochondria in these different cells. Mitochondria, however, differ slightly in their solubility in acetic acid and in other respects, and in direct proportion to the extent of variation the above argument loses force.

[1] This work was also carried on at the Marine Biological Laboratory, Woods Hole, Mass., where the Director, Dr. Lillie, very kindly placed a research room at my disposal. It was aided by the Carnegie Institution of Washington.

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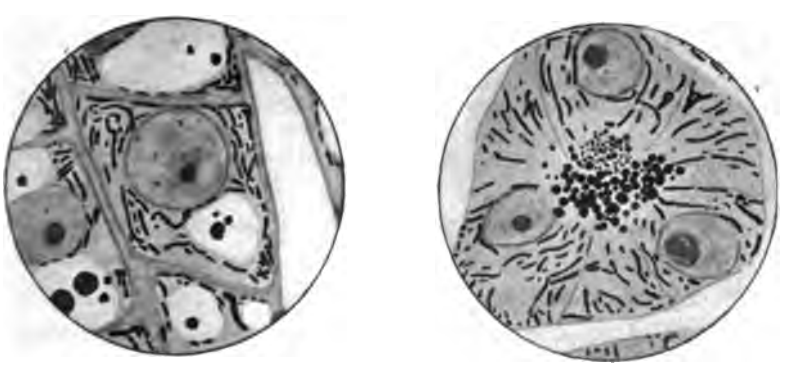
A very important issue at stake is the relationship of plant and animal mitochondria. Are the researches of the botanist of interest to the anatomist and vice versa? Should we attempt to coördinate and correlate the two, and, if so, how far can we go? Though these questions have never been directly attacked, considerable difference of opinion is apparent in the literature. Suffice it to say that Pensa ('14, p. 22) states that the mitochondria in plant and animal cells are not identical; Guilliermond ('13*a*, p. 481) and Smirnow ('06, p. 153) think that they are; Duesberg and Hoven ('10, p. 99) believe them to be homologous; while Sapěhin ('15, p. 320) holds an intermediate view. He thinks that in the higher plants mitochondria-like forms may be divided into two groups: "plastids" and "chondriosomes" which, however, are indistinguishable in the early meristem.

I have devoted my whole attention to this single problem of the relationship of plant and animal mitochondria with the hope of obtaining results which are clear-cut, concise and definite, bringing to bear upon it old, as well as entirely new, methods of technique in the form of supravital dyes of the janus green series which have never before been applied to plant cells.

Observations.

The statements concerning the similarity or the dissimilarity of plant and animal mitochondria have been made, with but few notable exceptions, by investigators having personal experi-



Figs. 1 and 2. Cells from the pea and the pancreas fixed in formalin and bichromate, mordanted in bichromate (Regaud IV. *B*) and stained with iron hematoxylin. (1,500 diameters.)

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ence with one or the other, seldom with both. It is essential that the two should be compared side by side.

I have chosen for my experiments the cells of the radicle of the pea and the acinus cells of the pancreas of the mouse on account of the close resemblance of their mitochondria. The general appearance may be seen by reference to Figs. 1 and 2. Morphologically the mitochondria would seem to be identical in the two; but in reality this is not the case, for I have examined them very carefully with a high magnification (3,500 diameters) and I find that they are, on the whole, slightly longer and thicker

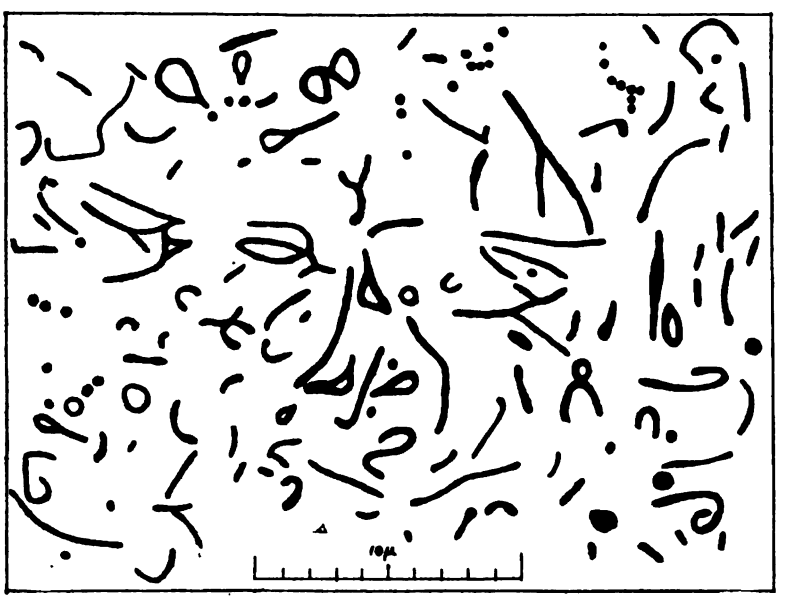

FIG. 3 Selected mitochondria from cells of pea radicle prepared by Regaud IVB to show the extent and limits of the polymorphism. (3,500 diameters.)

in the pancreas. I have made detailed drawings to scale of selected mitochondria from both. This can be done with considerable accuracy by making use of a good camera lucida, with appropriate illumination, and a sharply pointed pencil. The results are illustrated in Figs. 3 and 4. Filaments, rods and granules predominate. Branching filaments, networks, spherules and so on are rarer, but occur in both. In fact no form, however bizarre, is to be found in the one for which a counterpart cannot

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be discovered in the other. The absence of very minute mitochondria, merging into the invisible, is of interest from the point of view of an origin *de novo* through condensation; and the polymorphism, in both the pea and the pancreas, makes plain the hopelessness of any attempt to devise a system of individual nomenclature, based upon morphology, to embrace all the forms. Obviously the material selected permits of very close comparison.

I have taken precautions in the following experiments to eliminate, as far as possible, chance variations due to incurrent

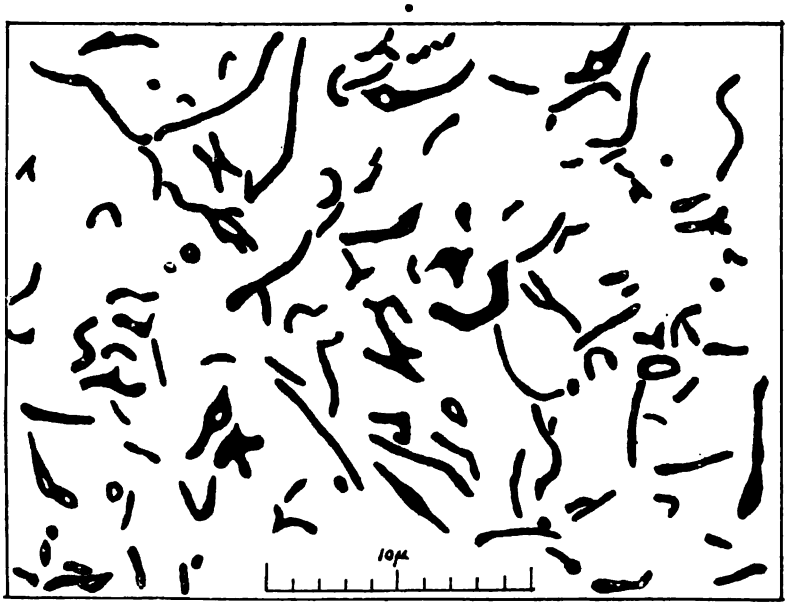

FIG. 4. Selected mitochondria from acinus cells of the mouse, treated in exactly the same way, showing similar polymorphism. (3,500 diameters.)

experimental errors which would, however, in any event, be likely to effect both tissues similarly (see p. 207). They were fixed together in the same bottle, dehydrated together, cleared together and embedded in the same block of paraffin together. They were cut with the same sweep of the knife, mounted on the same slide and stained at the same time. Many preparations have been made and in each of them the dehydration, clearing and embedding were carried on in the same way, so that the results are comparable. The sections were stained with iron

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hematoxylin, with fuchsin methyl green, with Altmann's fuchsin picric acid and sometimes by the Benda method. The chief results are briefly set forth in the following table, but a large number of additional controls were made.

In a general way, running our eyes down the table, we see that the response of mitochondria to fixatives is the same in plants as in animals. Similar fixations preserve them, modify them and destroy them in much the same way in both. It also shows that the technique is easy, not difficult, and that a number of chemicals, which are generally thought to be destructive, will fix them more or less satisfactorily.

The standard mitochondrial fixatives, like Regaud ('10, p. 296) II., III., IV. *A* and IV. *B*, Benda's modification of Flemming's fluid, the acetic acid osmic bichromate mixture of Bensley, and Zenker, without acetic, will fix them in both tissues. I have found, however, that Regaud's mixtures are by far the most satisfactory because they do not produce the artificial coagulation of the ground substance caused by the others. They also preserve the true form of the mitochondria more faithfully; though I have reason to suspect that in some cases, in plant cells, they cause fragmentation and shrinkage. I have studied the behavior of the ingredients of these fluids.

Formalin is perhaps the most important since it penetrates rapidly, retains the homogeneous appearance of the ground substance and preserves the mitochondria satisfactorily. Formalin alone (Sapěhin, '15, p. 321) serves as an excellent fixative for mitochondria. My best results were obtained with a solution of formalin of from 5 to 10 per cent. made up from ordinary commercial formalin neutralized with magnesium carbonate. More dilute solutions tend to cause a swelling of the mitochondria and more concentrated ones a shrinkage; but different tissues require different concentrations. Regaud advises a subsequent mordanting with bichromate, but I find that this is quite unnecessary. Used in combination with 3 per cent. potassium bichromate, according to the directions of Regaud, formalin gives the best of fixations with both the pea and the pancreas, though in my experience the results may be even improved by diluting the mixture with an equal volume of water as advised

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TABLE I.[1]

No.	Fixative.	Radicle of Pea.	Pancreatic Acini.
1615B..	5% formalin 41 days	+normal	+normal
1574A..	5% " 4 "	+normal	+normal
1575A..	10% " 4 "	+normal	+normal
1616A..	40% " 4 "	+irregular, shrunken	+nodular, shrunken
1580....	2.5% " 4 " mordanted in 3% bichromate for 21 days	+but slightly swollen	+swollen and fragmented
1574....	5% "	+normal	+normal
1575....	10% "	+fragmented	+few
1576....	20% "	+normal	+few
1616B..	40% "	+normal	+normal
1585....	formalin 10 c.c., saturated aq. picric acid 40 c.c. (Regaud II) 1 day	+fragmented	+normal, indistinct.
1585B..	same, mordanted in 3% bichromate 20 days (Regaud III)	+normal, unusually filamentous	+normal, indistinct
1577A..	5% formalin, 10 c.c., sat. aq. picric acid 40 c.c., 1 day	+fragmented, distorted	+fragmented
1577....	same, mordanted in 3% bichromate for 16 days	+fragmented, distorted	—
1578....	10% formalin, 15 c.c., 1% chromic, 85 c.c.; mordanted in pyroligneous 1 day and 2% bichromate 1 day	+fragmented	+few and fragmented
1566....	formalin 20 c.c., 3% bichromate, 80 c.c.; mordanted in 3% bichromate 15 days. (Regaud IV*B*)	+normal	+normal
1628 ...	Formalin 10 c.c., sat. aq. corrosive sublimate 40 c.c. 1 day	—	—
1629....	Formalin 10 c.c., sat. aq. corrosive sublimate 20 c.c., sat. aq. picric acid 20 c.c. 1 day	—	—
1582....	Boiling water 5 min.	—	—
1583....	95% alcohol	—	—
1447....	Gradual increase in concentration of alcohol, 2.5, 5, 7.5, 10, 15, 20, 30, 40, 50, 70, 80, 95% and absolute; chloroform	—	—
1584....	95% alcohol 10 c.c., 3% bichromate 10 c.c. 4 days	—	—
1618....	2% osmic 2 c.c. 2.5% bichromate 8 c.c., acetic 1 drop	+indistinct	+normal
1609....	sat. alcohol sol. corrosive sublimate	?	—

"+" signifies present, "—" absent and "?" doubtful.

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TABLE I—*Continued.*

No.	Fixative.	Radicle of Pea.	Pancreatic Acini.
1610....	sat. aq. corrosive sublimate	+distinct, shrunken	+few, indistinct
1611A..	sat. aq. picric acid	+nearly normal	+few, fragmented
1612....	2% chromic acid	+somewhat distorted	+few, fragmented
1613....	3% bichromate	+normal	+nodular rods, rings, granules
1614....	.5% osmic acid	+unsatisfactory	+normal

by Sapěhin ('15, p. 321). Formalin in combination with picric acid is a somewhat erratic fixative, very excellent in some instances and bad in others. In good preparations the mitochondria appear very large and not so shrunken as when acted on by other fixatives. Formalin and chromic acid constitute a poor fixative which fragments the mitochondria and, in some cases, destroys them entirely.

Potassium bichromate used alone (Sapěhin, '15, p. 321) is a good preservative for mitochondria but penetrates badly. Regaud recommends its use in mixtures and as a mordant in order to render the mitochondria insoluble in the alcohols during dehydration. The bichromate which remains in the tissue when it is sectioned, increases its affinity for fuchsin when the fuchsin methyl green stain is applied.

Ethyl alcohol in 95 per cent. solution destroys the mitochondria in both the pea radicle and the pancreas. When alcohol is employed in gradually increasing concentration the same result is obtained. When it is combined with potassium bichromate the mitochondria are also destroyed. In fact our conclusion can be none other than that alcohol, in whatever combination, is a very poor fixative for mitochondria.

Boiling water preserves the general form relations but does not bring to light any mitochondria.

Picric acid is a fairly good fixative for mitochondria in the cells of the pea and pancreas, when used in a saturated aqueous solution. It has the great advantage that it penetrates well.

Chromic acid alone causes considerable distortion of the mitochondria and somewhat irregular coagulation of the ground substance but in combination it is apparently very efficacious as shown by its inclusion in Benda's modification of Flemming's fluid and in other mixtures.

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Osmic acid is the best preservative of mitochondria and the worst penetrator and is therefore useless in the cells of the pea radicle on account of their impermeable cellulose walls; yet it is this quality of being an excellent preservative which makes it so valuable in association with other chemicals. It does not cause any artificial coagulation of the ground substance.

Corrosive sublimate in saturated aqueous solution also penetrates the pea radicle very poorly; the pancrease not so badly. However it preserves the mitochondria in the pea far better than in the pancreas in spite of the fact that they are, in both cases, somewhat shrunken. In alcoholic solution it is destructive, but it is difficult to tell whether this is due to the alcohol or to the increased amount of sublimate. In Zenker's fluid the injurious action of the sublimate is counter-balanced by the presence of potassium bichromate.

The action of acetic acid is so important that I have made a detailed study of it as set forth in Table II.

In the first place I have varied the concentration of acetic acid in Regaud's mixture IV. *B* from 0 per cent. to 10 per cent. With no acetic acid the mitochondria are normal; while with a concentration of 2.5 per cent., or more, they are destroyed in both tissues, showing that their degree of solubility is the same.

I have also experimented with Zenker's fluid and I find that, with it, the mitochondria are on the whole much more resistant to acetic acid. This may be due to the fact that the Zenker's fluid was only applied for one day, instead of for five days like the Regaud's fluid; or the presence of sublimate in the Zenker's fluid may have tended to counteract the action of the acetic acid. The mitochondria are fairly well preserved when no acetic acid has been added to the Zenker's fluid. With increase in acetic acid, up to 10 per cent., they become destroyed in the deeper parts of the tissue, especially in the pea; while they are fairly well preserved in the most superficial cells in both. In the pea, also, the mitochondria are far more prone to become fragmented. This difference between surface and interior seems to be a question of penetration rather than of a difference in the mitochondria themselves. There is a simultaneous action of all the ingredients of the fixative on the cells at the surface and here the

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TABLE II.

No.	Fixative.	Radicle of Pea.	Pancreatic Acini.
1570....	Zenker 0% acetic 1 day	+better preserved in older cells, apt to be vesicular, fragmented	+normal
1571....	" 2.5% "	+better preserved in older cells, apt to be vesicular, fragmented	+normal in peripheral cells, absent in center of tissue
1572....	" 5% "	+almost all destroyed in younger cells, still fairly numerous in older ones	+fewer, only in peripheral cells slightly fragmented
1573....	" 10% "	+more fragmented and vesicular	+same
1617....	" 20% "	+fairly well shown in all cells	+slightly fragmented, present in all cells
1627....	" 33% "	+but very rare	+but very rare
1626....	" 50% "	—	—
1566....	Regaud IV*B*, 0% acetic 5 days, mordanted in 3% bichromate 8 days	+normal	+normal
1567....	Regaud 2.5% acetic 5 day	—	—
1568....	" 5% "	—	—
1569....	" 10% "	—	—
1625....	Equal parts acetic and water	—	—
1624....	Pure acetic	—	—
1565....	Benda no acetic 8 days	+rather vesicular	+normal
1579....	Benda, 0.8% acetic, mordanted in formalin 20 c.c.; 3% bichromate 80 c.c. 6 days	+normal few	+normal
1579B..	Same but mordanted in pyroligneous 1 day and 2% bichromate 2 days	+normal few	+normal

bichromate, and possibly also the sublimate, modify the action of the acetic acid. In the deeper layers of the tissue, on the other hand, we have to do with a successive action of the said ingredients, determined by their relative rates of penetration. The acetic acid penetrates first and acts upon the mitochondria before the bichromate and the sublimate have come upon the scene.

Mitochondria are surprisingly resistant to higher concentrations. They are well preserved by a mixture of Zenker's fluid and 20 per cent. acetic acid and their relations are shown in Figs. 5 and 6. With 33 per cent. acetic acid only a few scattered mitochondria remain, while they are completely destroyed by a mixture containing 50 per cent. of acetic acid.

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Pure acetic acid in half concentration and in full concentration destroys the mitochondria in both tissues. But it must not be thought that acetic acid in small concentrations is to be avoided because I have found that Benda's fluid without acetic acid does not seem to act so well as with acetic acid, in the amount prescribed.

In other words acetic acid exercises a solvent action on mitochondria which is the same in plants as in animals and varies with the distance of the cells from the surface and with the

FIGS. 5 and 6. Cells from the pea and the pancreas fixed in Zenker's fluid containing 20 per cent. of acetic acid and stained with fuchsin and methyl green. (1,500 diameters.)

character of the other components of the fixative. There is reason to believe, further, that the resistance of mitochondria in certain young plant cells is less than in old ones, calling to mind the condition which obtains in the spermatogenesis of many animals where the mitochondria in the young cells are more susceptible to acetic acid than in the mature spermatozoa (Regaud, '08, p. 661); which comparison makes the resemblance of plant and animal mitochondria, in this respect, still closer.

I have attempted to ascertain the experimental error in the study of mitochondria in fixed tissues in plants and animals and to see whether it is the same in both.

The conditions of the experiment are not so important as the nature of the fixative but they must nevertheless be taken into consideration. Variations in the temperature (under say 46° C.) and the illumination of the tissue prior to fixation do not seem to influence the mitochondria for I have grown peas at room

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temperature and in the refrigerator (8 to 12° C.); in bright light and in darkness without bringing about any noticeable change in their mitochondria. Some fixatives are rather unstable and cannot be kept, even for a few hours, in bright sunlight or in a warm place. Under these conditions Regaud's formalin and bichromate mixture, for example, undergoes a rapid change characterized by a darkening in color, but its action does not seem to be impaired, though, to be on the safe side, one should avoid it. On the other hand, mechanical manipulation of the tissue before fixation often causes very confusing alterations in the mitochondria especially in the softer animal tissues.

The distribution of mitochondria within the cell is not altered appreciably in either plants or animals by the technique used, except in instances where mitochondria are present with different solubility, some being preserved and others being destroyed. This is of common occurrence in the pea radicle where with some fixatives, filaments only appear; the granules being obliterated so that the apparent distribution is altered.

The technique makes a very great difference in the number of mitochondria in the preparation. Some ingredients of the fixative are particularly likely to destroy them, like the acetic acid already mentioned; so that in the study of unfamiliar tissues we must assure ourselves that the technique is adapted to show all the mitochondria present.

The shape of mitochondria is so easily modified by the fixative that we must be on our guard here also, especially in plant cells. That is to say, we frequently meet with a fragmentation of mitochondrial filaments, the filaments of the living cell appearing in the form of rows of granules in the fixed preparation. The reverse change never takes place, for filaments are never formed through a coalescence of granules, under the influence of the fixative. Poor penetration of the fixative in the deeper layers of the tissue often causes the mitochondria to lose their characteristic form and to swell up into large spherules or vesicles. Similarly if a film on the surface of the tissue is allowed to dry, before fixation, the mitochondria in it will be profoundly modified.

The size of mitochondria is also subject to some modification, as shown by the comparison of mitochondria in living cells with

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those in fixed tissues. The general tendency of fixatives is to shrink them and to make them uniformly smaller than they really are in the living condition. This tendency is very slight, almost negligible, in the case of the best fixatives. On the other hand, acetic acid, and possibly also formalin in very low concentrations, have a marked swelling effect.

It must be admitted that variations sometimes do occur in the mitochondria in our preparations, particularly in the pea, for which no reasonable explanation can be advanced on the basis of the technique. They may result from rhythmical variations in the activity of the cells which we have reason to believe occur, just like periodicity in cell division, many cells dividing almost synchronously (Kellicott, '04, p. 531) but they cannot be wholly explained in this way. They may result also from the conditions under which the radicle is growing, upon whether it is submerged or exposed to the air; but I find that radicles grown under exactly the same conditions occasionally show mitochondrial variations which are very perplexing.

Evidently the experimental error is something which we must have considerable respect for, but which can be controlled if the proper precautions are taken. Its identity in plant and animal tissues is another indication of the similarity of the mitochondria in the two.

There is another factor, often very confusing, which we have to bear in mind, namely that of the production of coagula in the homogeneous ground substance. They are formed in the protoplasm of the pea radicle by exposure for about 30 minutes to a temperature of 48° C. or higher and also by fixatives containing acetic acid, chromic acid, corrosive sublimate or alcohol. Iron hematoxylin, when imperfectly differentiated with iron alum solution, stains these coagula in the same manner as the mitochondria and in many cases no clearly marked distinction can be made between them, since they both vary very much in their resistance to differentiation. The formation of these coagula is generally accompanied by distortion, fragmentation or destruction of the mitochondria. In the cells of the pancreas these fixatives bring about a distinctly fibrous appearance in the normally homogeneous basophilic substance which is often as re-

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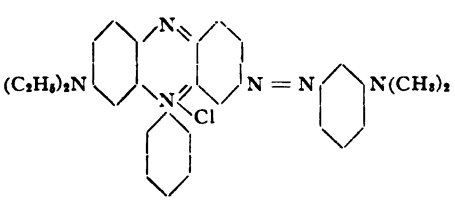
sistant to differentiation as are the mitochondria, but the destructive effect is not so apparent as in the cells of the pea radicle.

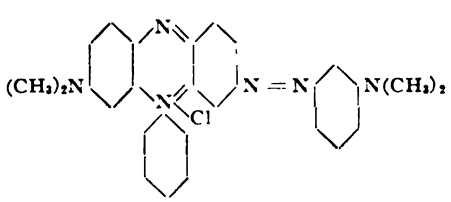
The question of the reactions of plant and animal mitochondria. after fixation, to stains may be dismissed with very few words, When they have been fixed by any method, it is usually possible to color them with any stain, by the Benda method, with fuchsin-methyl-green, with fuchsin-picric acid or with iron hematoxylin, but it is sometimes advisable to select some mordant in order to induce the stain to take. The Benda method is the most difficult and it is sometimes refractory, for no apparent reason, in tissues where the others give excellent results. In other words there is nothing whatsoever specific about the staining reaction.

A number of supravital stains have been discovered for mitochondria in animal cells, of varying degrees of specificity, and it is interesting to note that none of them have heretofore been applied to plant mitochondria. The most delicate is the janus green reaction, which we owe primarily to Michaelis ('99, p. 565). The delicacy of this reaction is shown by E. V. Cowdry's ('16, p. 429) observation that janus green B will stain mitochondria specifically in human lymphocytes in a dilution of half a million (*i. e.*, one part of stain to 500,000 parts of salt solution), and that janus green G will not stain them even in a relatively strong solution.

Janus green B is diethylsafraninazodimethylanilin:



Janus green G is dimethylsafraninazodimethylanilin:



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Janus green G differs only in the substitution of a *dimethyl* group in the place of the *diethyl* group in the safranin molecule. Janus blue and janus black I were first introduced as vital stains for mitochondria by E. V. Cowdry ('16, p. 431). Janus blue is the trade name for diethylsafranin-B-napthol and janus black I is a mixture of diethylsafraninazodimethylanilin and some other dye of unknown constitution. Diethylsafranin may easily be made from janus green by splitting off the azodimethylanilin group (Cowdry, '14, p. 269). I have applied these dyes to the mitochondria in the cells of the pea radicle and the acinus cells of the pancreas with the following results.

TABLE III.

Dye.	Cells of Pea Radicle.	Acinus Cells of Pancreas.
Janus green B	+ Intense	+ Intense
Janus green G	−	−
Janus blue	+	+
Janus black I	+	+
Diethylsafranin	?	? + faint
Nilblue B, extra	+ faint	+ faint

It will be seen that the mitochondria in plant cells react to vital stains in precisely the same way as in animal cells, even to this extraordinarily delicate janus green test. It is very much more difficult, however, to get a good stain in plant cells by reason of their tough, cellulose walls offering an almost insurmountable barrier against the penetration of the dye; and janus green is at best but a poor penetrator compared with neutral red, methylene blue and others. For this reason the staining of mitochondria is much slower than in animal cells. Great difficulty was also experienced in finding a suitable medium for the examination of the two tissues. At first they were examined, side by side, in weak aqueous solutions of the dyes to which sodium chloride had been added in varying amounts. This proved entirely satisfactory for the pancreas, but failed with the pea, for which a sugar solution was used. Another trouble with the pea, not encountered with the pancreas, is that the cells are cemented closely together and are therefore so very difficult to separate by teasing, that thin sections of the living pea radicle had to be cut with a razor blade. This method also is objection-

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able because a comparatively large amount of the tissue juice is liberated which brings about a troublesome precipitate of the dye and makes it necessary to renew it frequently. In short, plant cells are very hard to handle but the stain is none the less specific. The petals of some flowers (narcissus, sweet pea, freesia, etc.) give very much more satisfactory results than the pea since with them the dyes penetrate more easily.

I have also tried to stain the living plants *in toto* by growing them in sand moistened with a strong solution of janus green B. I discovered at once that they react differently to this treatment. Peas were stunted, but grew and flowered without any coloration with the exception of the root hairs and small portions of the epidermis which seemed to be dead. On the other hand, gourds grew vigorously for a time and became intensely stained while growing. They presented a very beautiful appearance because in some of the tissues the dye is reduced to its color base, diethylsafranin, so that the plant is marked off into red and bluish green areas following the distribution of processes of reduction and of oxidation. Cells from the different regions were examined and it was found that the whole protoplasm was stained more or less uniformly and that the mitochondria were not specifically colored. These experiments would seem to indicate that the vital staining of plants with the less toxic azo dyes is full of promise from the point of view of plant physiology.

This specific comparison of the mitochondria in the pea and in the pancreas has shown that morphologically they are almost identical, save for a slight difference in diameter. No forms are present in the one for which counterparts cannot be discovered in the other. If they could be viewed dissociated from their environment it would be next to impossible to tell which belonged to the pea and which to the pancreas. Microchemically they are identical so far as our imperfect methods go. Similar fixatives preserve them, modify them and destroy them in like fashion in both. Even the experimental error is the same. Finally, and most important of all, they react the same way to the janus green test and to other supravital dyes. Accordingly our provisional conclusion can be none other than that the mitochondria in the pea and in the pancreas are composed of precisely the same material.

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But this is only a single isolated instance and many facts of interest can be brought to light by a broad general discussion of mitochondria in many forms which I shall now venture to make, based in part upon the literature.

Discussion.

Distribution.

In plants mitochondria have been recorded from the *Angiosperms* to the *Fungi;* but it is difficult or even impossible to demonstrate their existence in the very lowest forms of plant life, like the *Myxomycetes*, the *Schizomycetes* and some of the *Algæ;* though in these groups structures of questionable nature have been discovered which may ultimately prove to be mitochondrial. This absence of typical mitochondria in the lowest plants may be contrasted with their almost universal occurrence in the *Protozoa*. In all forms of animals, from amœba to man, which have been investigated with adequate methods of technique, they occur without exception.

With regard to the different types of cells. In plants, they occur from the tip of the root to the end of the stem, wherever the protoplasm is active, with but few exceptions. The same is true in the various categories of animal cells. They are met with in gland cells, nerve cells and muscle cells; in connective tissue cells, germ cells and almost all others, except in the terminal stages of cytomorphosis. And this is one of the greatest points of similarity between these granulations in the plant and animal kingdoms, that it to say their progressive diminution and final absence in the later stages in the life of the cell.

I refer, for instance, to the decrease in number of mitochondria in plant cells, which runs parallel to the formation of chloroplasts, for it is said (Guilliermond, '12, plates 17–18) that when the plasts are fully mature few if any mitochondria remain (which reminds one of the single large chloroplast and the absence of mitochondria in some algæ); and these are mature and highly differentiated cells. In animals there is a similar disappearance of mitochondria in the life cycle of red blood cells. In the young, nucleated forms they are very abundant, they become less and less so as the cell differentiates; a few persist after the nucleus is

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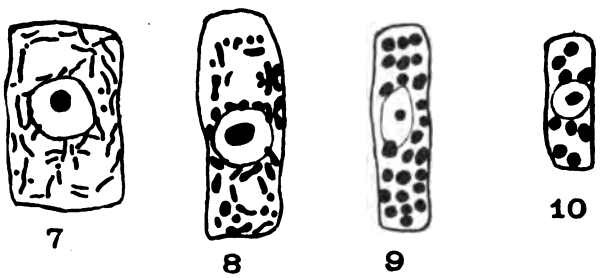
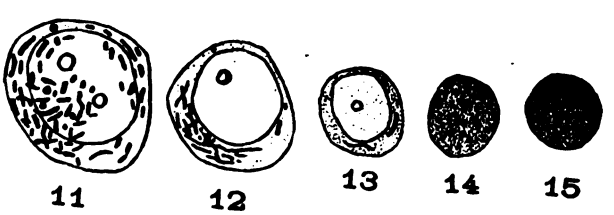
lost; but in the fully mature forms, as they occur in the circulation in man, mitochondria are entirely absent. In plants this disappearance is associated with the production of chlorophyll; in animals, with the formation of hemoglobin, two substances with strikingly similar chemical constitution; in both it is progressive and runs parallel with an increase in the degree of



FIGS. 7, 8, 9 and 10. Meristem and parenchyma cells of the bean (after Guilliermond, '12, plate 18, modified) showing the progressive disappearance of mitochondria with the formation of plastids containing chlorophyll.

differentiation and with the age of the cell, general metabolism being diminished and special functions being accentuated (Figs. 7–15).

This diminution of mitochondria in cytomorphosis is really of very common occurrence but it attracts attention only in those



FIGS. 11, 12, 13, 14 and 15. Erythroblast, megaloblast, normoblast and erythrocytes from bone marrow of a rabbit stained vitally with janus green showing the parallelism between the disappearance of mitochondria and the appearance of hemoglobin in the form of a diffuse deposit. (1,500 diameters.)

cells which normally die and are replaced in large numbers, collectively, in the life of the organism.

In the separate regions of the cell, also, there is a general similarity in the distribution of mitochondria in plants and animals. Where the cells are elongated, as in the plerome,

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filamentous mitochondria are usually distributed parallel to the long axis; which calls to mind the arrangement of mitochondria in gland cells, nerve cells, muscle cells and others. In the serous cells of the parotid they are heaped up in the proximal region next the basement membrane and remote from the lumen (Fig. 16). Where the polarity is reversed as in the thyroid (Fig. 17), the mitochondria are also reversed; while in the epithelial cells of the intestine, they are condensed at both poles (Fig. 18). This, Champy ('12, p. 109) thinks, is indicative of a double polarization for adsorption and for secretion. Corresponding condensations have not been recorded in plant cells.

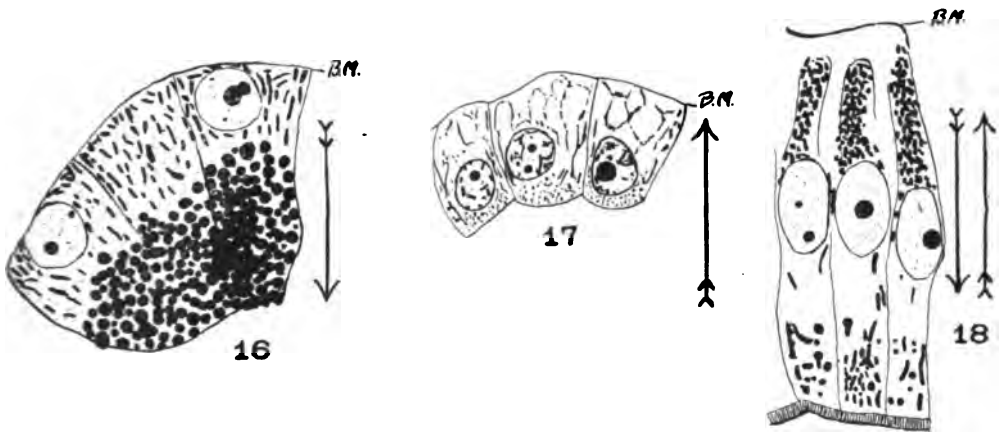

FIG. 16. Serous cells of parotid of mouse with proximo-distal polarity, as represented by the arrow, B. M. being the basement membrane. (1,500 diameters.)

FIG. 17. Thyroid cells (after Bensley, '16, p. 55) with reversed polarity, disto-proximal, in the direction of the basement membrane.

FIG. 18. Intestinal epithelial cells with double polarity, mitochondria being accumulated at both poles. (1,500 diameters.)

Mitochondria, however, group themselves about the nucleus in both plants and animals. In the early meristem of plants, generally, mitochondria are found indifferently distributed in the protoplasm (Fig. 19). They soon approach and appear to come into actual contact with the nucleus (Fig. 20) in which position they enlarge and form plasts which migrate away from the nucleus and become distributed more or less evenly in the surrounding protoplasm (Fig. 21). Guilliermond has repeatedly described this migration. I find that the mitochondria become

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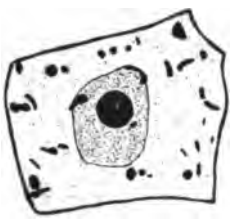
progressively more resistant to acetic acid in this process of plast formation. Similarly in the spermatogonia of certain animals the mitochondria are diffusely arranged (Fig. 22); in the spermatocytes they approach the nucleus (Fig. 23) and become so closely applied to it that investigators have been deluded into thinking that they actually originate from it. In the later stages of spermatogenesis they leave the nucleus (Fig. 24)



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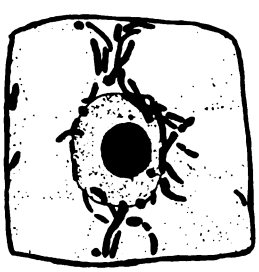

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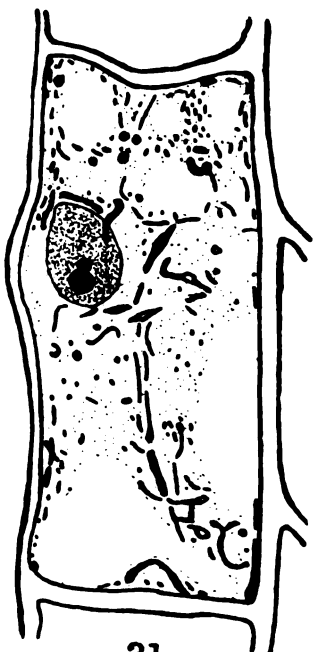

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FIGS. 19, 20 and 21. Meristem and young and old cortical cells of the pea fixed in formalin and picric acid (Regaud III) and stained with iron hematoxylin showing primary diffuse arrangement of mitochondria, secondary condensation about the nucleus and final even distribution throughout the whole cytoplasm. (1,500 diameters.)

becoming more resistant to acetic acid as Regaud ('08, p. 662) has shown. Indeed the parallelism is very close. Other instances might be cited.

In plant cells one rarely finds a condensation of mitochondria in the peripheral cytoplasm though it is frequently met with in animal cells. It is particularly manifest in certain stages in the

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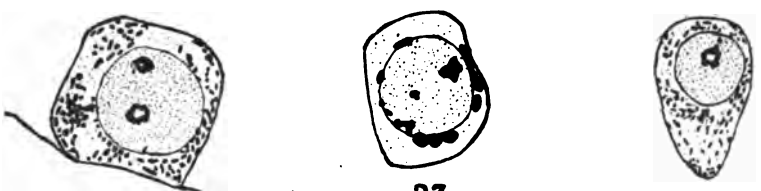
growth and maturation of the egg as Van der Stricht ('09, plate 1) and his pupils, among others, have abundantly shown. The causes underlying these strange movements of mitochondria are obscure.

Furthermore, during cell division the mitochondria are distributed in much the same way in plants as in animals. They persist during the whole process; they are absent in the spindle area, whether a definite spindle be formed or not; and they are divided in approximately equal amounts between the two daughter cells.



22 23 24

FIGS. 22, 23 and 24. Spermatogonium, spermatocyte and spermatid from mouse testis fixed in formalin and bichromate (Regaud IV*B*) and stained with iron hematoxylin. Note diffuse arrangement of mitochondria, circumnuclear condensation and final diffuse arrangement. (1,500 diameters.)

In animal cells they are almost invariably disposed in a radial fashion about the centrosome: contrasting strongly with their appearance in *Pustularia* during spore formation when they are entirely absent from the region near the centrosome and are found in a clump in the portion of the cells farthest away from it (Guilliermond, '13*b*, p. 649). No examples of a radial arrangement have to the best of my knowledge been described in the higher plants but this may be due to the well-known absence of a typical centrosome in the *Angiosperms*.

It will be noticed that in animal cells rather more variations obtain in the arrangement of mitochondria than in plant cells, but this seems to be correlated in some way with the fact that animal cells are more generally polarized. I mean polarized for irritability, conduction, secretion, contraction and so forth, properties which do not play so great a rôle in the life of plants, where separate regions of the cell are not so distinctly marked off. Accordingly if one were searching for variations in the distribu-

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tion of mitochondria in different parts of plant cells one would be inclined to examine cells polarized with reference to light, secretion and so on, where cytoplasmic division of labor may be expected.

Morphology.

As we pass down the plant scale we find no noteworthy differences in the morphology of mitochondria, even in those *Algæ* which possess them they are alike. Similarly, few animals have mitochondria of distinctive morphology, though in certain species of both plants and animals, either filamentous or granular forms may predominate.

In the different tissues of plants there is some variation in the size and shape of mitochondria. In some cells thick filaments are most abundant, while in other kinds of cells, granular forms of variable size may be found. Networks are rarely met with. The question is complicated by the fact that in certain tissues all morphological transitions between typical mitochondria and true plastids are to be seen; swellings develop in the region of the mitochondrial filaments, which apparently grow larger and larger and ultimately form mature plastids.

In the different tissues of animals there is rather more variation in the morphology of mitochondria. They are usually filamentous in gland cells, rod-like in muscle cells and they are often granular in egg cells. Networks occasionally occur in the pancreas and in spermatogonia as well as in other locations. Even within gland cells of the zymogenic type there is considerable variation. The mitochondria in the acinus cells of the pancreas are uniformly longer and thicker than those in the chief cells of the fundus of the stomach and I find that they are two or three times as long as those in the serous cells of the parotid of the mouse (compare Figs. 2 and 16). The mitochondria in zymogenic cells often possess little enlargements which are supposed to be the precursors of granules of zymogen, and which call to mind the swellings on the mitochondria in plant cells during plastid formation. The similarity of the process may be seen by taking two specific instances; the production of metachromatic corpuscles in plants as figured by

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Guilliermond ('13*c*, p. 438) and the formation of fat as described by Dubreuil ('13, p. 104*B*). Compare Figs. 25 and 26.

Striking differences also obtain in the morphology of mitochondria in the different categories of nerve cells. Nicholson ('16, p. 347) has found that they are usually filamentous, especially in the anterior horn cells and in the cells of the reticular formation; they are rod-like and granular in the large and small cells of the Gasserian ganglion, and they occur in the form of large irregular blocks in the cells of the trapezoid nucleus.

25 26

FIG. 25. Metachromatic corpuscles developing in mitochondrial filaments (after Guilliermond, '13*c*, p. 438).

FIG. 26. Droplets of fat forming in mitochondrial filaments (after Dubreuil, 13, p. 104).

The greater polymorphism of animal cells, with regard to their mitochondrial content, probably results from the fact that their functions are more diversified, for they have a greater variety of duties to perform under different conditions. Apparently their distribution within the cell is more varied in animals than in plants for the same reason (p. 212). The form of mitochondria is apparently independent of the degree of mobility of the surrounding protoplasm.

It is worthy of note that in developing tissues of both plants and animals similar and parallel changes take place in the morphology of mitochondria, at least in certain instances. In the spores of plants and the egg cells of animals, they are usually granular and sometimes filamentous; in the developing tissues of the embryo they are usually filamentous and rarely if ever granular. With the assumption of special functions on the part of the cells in the different organs they change their form. Some remain filamentous and acquire blebs (secreting cells), others become more rod-like (muscle), still others granular, and so on.

A single cell may contain all the types of mitochondria which have been enumerated. This is true for animals as well as for

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plants. Generally, however, filamentous, rod-like, dumb-bell and granular forms are met with. Filaments, when present, have an astonishingly uniform diameter in the same cell, though they may possess the swellings already mentioned. Segmentation of filaments is of common occurrence in both and it is possible that a coalescence of granules may take place. In some tissues there is considerable variation from cell to cell, while in others the morphology of the mitochondria is quite uniform throughout. Thus the "anthocyane" producing mitochondria, figured by Guilliermond ('13*a*, p. 479), are uniform; those in the cortical cells of the pea radicle, variable in length. In neighboring acinus cells of the pancreas of the mouse they are uniform; in contiguous spinal ganglion cells, variable.

A fundamental distinction may be made between variations in length and variations in breadth, in the case of both plant and animal mitochondria, to which I have seen no reference in the literature. While the mitochondria in the cells of the pea radicle vary greatly in length, filamentous and rod-like forms predominating, their girth is remarkably uniform, throughout the whole tissue, in individual cells and in different parts of the same filament (Fig. 1). The same holds for the pancreas (Fig. 2). The uniformity in girth extends right to the end of the filaments which always end abruptly without tapering. The truth of this statement is made still more apparent by examining any illustration of mitochondria, because artists are wont, quite rightly, to draw each filament with a single sweep of the same pen or brush. The one great exception to this rule is plastid formation in plants and the production of bleb-like swellings in association with secretion in animal cells. The diameter of the mitochondrial filament is fixed, but the length is not; one is stamped on the cell through its organization and is probably dependent on the water content of the surrounding cytoplasm, the other is most likely an expression of growth by accretion affecting the length but not the diameter. We have here two attributes, independently variable, which may perhaps be influenced in different ways, common to both plant and animal mitochondria, which speak more strongly for their identity than any other.

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Composition.

We must bear in mind the unsatisfactory nature of the evidence upon which our views of the chemical constitution of mitochondria are based. It is impossible to make a direct chemical analysis of them. We are forced to use solvents, special stains and so forth. We know astonishingly little positively, but we infer a great deal, often on very insufficient grounds. Most of the work has been done with animal cells, and investigators have arrived at the general conclusion that here mitochondria are of the nature of phosphatids, in other words, that they contain fatty acid, phosphoric acid, glycerol and some nitrogen base. Lecithin is an example of a typical phosphatid and has been used to manufacture artificial mitochondria. Briefly the evidence is as follows:

1. In animal cells it has been found that mitochondria are almost completely soluble in alcohol, ether, chloroform and dilute acetic acid. They are rendered insoluble by chromization, and also, in my experience, by treatment with formalin, at least in some cases. They do not stain with Sudan III. or Scharlach R. They are only sometimes blackened with osmic acid.

In plant cells they are also soluble in alcohol, ether, chloroform and dilute acetic acid and they are here rendered insoluble by chromization in exactly the same way. Treatment with formalin also makes the mitochondria insoluble. The whole cell blackens with osmic acid so readily that it is impossible to ascertain just how strongly the mitochondria themselves react to it.

2. It is said, in the literature, that part of the mitochondrial substance, in animal cells, is not soluble in these fat solvents and it has been assumed that this portion is albumin. The results of applying Millon's reagent are uncertain and difficult to interpret.

In view of the statement of Forbes and Keith ('14, p. 73) that vegetable phosphatids differ from those in animal cells in that they nearly always contain sugar firmly bound to the rest of the molecule, we should bear in mind the possibility of sugar being a likely constituent of mitochondria in plants.

3. Artificial mitochondria have been made by Löwschin ('13, p. 203) of lecithin and albumin solutions which apparently

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present the same form and solubilities as true mitochondria in both plant and animal cells. They form granules, rods and filaments which multiply by division. He embedded them in glycerin-gelatin, fixed them and found that they stained in the usual way by the various mitochondrial methods.

I have myself experimented with lecithin. I made a very fine emulsion, evaporated it to dryness on glass slides and stained with iron hematoxylin. The bodies which I observed resembled mitochondria in some respects but in others differed so very radically from them that I am unable to confirm Löwschin's observations.

4. The temperature solubility of mitochondria may be also significant because mitochondria, like phosphatids, are thought to have a low dissolving point. Policard ('12, p. 229) observed, in the case of some animal tissues, that the mitochondria are dissolved when subjected to a temperature of from 48° C. to 50° C. in a moist atmosphere from 10 to 30 minutes, while the other parts of the cells remain practically unaffected; and Koch and Voegtlin ('16, p. 59) remark that phosphatids are notoriously unstable to heat. With the hope that this temperature solubility would turn out to be a definite physical test for mitochondria, I experimented with both the pea and the pancreas, observing the precautions outlined on page 199. I found that in both the pea and the pancreas they dissolve at about the same temperature, that is to say, from 48° to 50° C., when treated for 30 minutes. The first change noted is a loss of their filamentous form (see also the Lewises, '15, p. 375); they became granular with indistinct outlines merging into the surrounding cytoplasm and they finally disappeared leaving no trace behind. In the case of the pea, the mitochondrial changes are difficult to make out on account of the production of a confusing coagulation of the ground substance.

5. The specific gravity of mitochondria is generally greater than that of the protoplasm in which they are embedded (Fauré-Fremiet, '13, p. 602). This is determined by the unsatisfactory centrifuge method. When they are thrown down they are said to be of high specific gravity. If the protoplasm is in the physical condition of a gel, rather than a sol, as in the nerve cell, the dis-

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tribution of mitochondria is unaltered by centrifuging, as Key discovered. But this is no reason to believe that these mitochondria differ from others in their specific gravity. At any rate, where the method is applicable (*i. e.*, in egg cells) the mitochondria are heavier than protoplasm, in which respect they conform to what we know of phosphatids and differ sharply from oils and neutral fats which rise to the surface and float, instead of being thrown down.

6. In some animal cells mitochondria act as solutes for different substances. Pigments of various kinds are frequently found dissolved in their substance so that they assume the most brilliant hues. Asvadourova ('13, p. 263), following Prenant, calls them "chromochondria" on this account. The presence of droplets of neutral fat within the mitochondrial filaments has also been recorded.

Plant mitochondria behave in exactly the same way, anthocyanin, chlorophyll and other pigments, sugar, starch and even fat being heaped up within them (Guilliermond, '13*b*, p. 647).

7. There seems to be a certain correspondence between variations in the histological picture of mitochondria and variations in the phospholipin content of the same organ on chemical analysis. Thus Mayer, Rathery and Schaeffer ('14, p. 612) have been able to alter the mitochondria experimentally in liver cells. In stages with more mitochondrial substance, chemical analyses show an increase in phosphorized lipoid; in stages with less, diminution.

Unfortunately mitochondria in plant cells have not been investigated from this point of view.

8. And, finally, the very interesting observations of Russo and Rene Van der Stricht must be mentioned. Russo ('12, p. 215) claims to have been able to increase the number of mitochondria in the oöcytes of the fowl by the injection of lecithin and Rene Van der Stricht ('11, p. 435) obtained results which seemed to be confirmatory.

Since there have been no observations along this line in plant cells I have attacked the problem by growing peas in solutions of lecithin. The results were in no sense definite and concise, though the mitochondria did seem to be increased in diameter.

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Physiology.

It is generally conceded that mitochondria in plant cells play an important part in the elaboration of chlorophyll and starch. They are thought to do this through the intermediary of the chloroplasts. According to Guilliermond ('12, p. 387) chlorophyll appears in typical mitochondria, increases in amount, other changes take place and the mature chloroplast results. It is not surprising, therefore, that mitochondria have attracted so much attention among European botanists, because the formation of these substances had been under discussion for nearly a century and a deadlock had been reached before these new mitochondrial methods were devised. Indeed the formation indirectly of starch from atmospheric carbon dioxide and water, through the action of sunlight on chlorophyll, is the most fundamental of all vital processes in plants. Mitochondria are concerned in the formation of chlorophyll and thus the very existence of the plant depends on them. Plants furnish the food of animals so that the importance of mitochondria with respect to the food supply is apparent.

Very obviously the mitochondria in typical animal cells can take part in no such process since there are no plastids, and starch is elaborated only in plants. But this is not, as it might seem to be, a fundamental distinction between the two, for many consider the animal mitochondria themselves to be plast-like and to act as such in the elaboration of secretions. The evidence for this, however, is not entirely conclusive and we must bear in mind, in all experimental work, the great importance of the homogeneous ground substance, or environment, in which the mitochondria are embedded. Apparent alterations may occur in the mitochondria which cannot be attributed to the mitochondria themselves, but only to changes in their environment. This is particularly true in the case of their refractive index. The mitochondria may stand out sharply in one cell and be quite invisible in another and yet be identical as far as their function is concerned in the two cells, for variations in the surrounding protoplasm may alone be responsible for the difference in appearance. There are thus two variables, the mitochondria and the protoplasm, either one of which may bring

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about a visible difference in the living cell independently of the other, though in the majority of the cases they probably act together. So that it is very difficult to tell whether the mitochondria are active or passive agents.

The belief is gaining ground (Kingsbury, '12, p. 46; Mayer, Rathery and Schaeffer, '14, p. 619, and others) that mitochondria are concerned with respiration in animal cells; that is to say, with the taking up of oxygen. This conception is based upon the view that mitochondria chemically resemble phosphatids, it being thought by some that phosphatids outside the body are capable of auto-oxidation. It falls well in line with the very wide distribution of mitochondria as well as with the fundamental nature of the process of respiration. And of course, the same arguments apply to mitochondria in plant cells for plant cells also take up oxygen. It seems that, in this particular, there is no great difference between mitochondria in these two great groups of organisms.

Unfortunately it is not possible to transfer plant mitochondria to animal cells to see what they would do, or vice versa. We are working in the dark, but we have only ourselves to blame because we have not taken full advantage of certain lowly protozoans, like *Euglena viridis* and others, which have, confined in the scope of a single cell, all the properties which we are prone to consider distinctive of both plants and animals. They contain chlorophyll, produce paramylum, a carbohydrate resembling starch, and at the same time engulf, devour and digest certain still more minute organisms. The brief description of mitochondria-like material in *Euglena*, made by Ternetz ('12, p. 463), would serve as a point of departure. The planarian worm, *Convoluta roscoffensis*, which takes in algæ and lives in symbiotic relationship with them would repay further study. We have here an animal containing animal mitochondria with all their attributes which, after symbiotic relationship has been established, "ceases to take in solid food and depends entirely upon its vegetable partners" (Bayliss, '15, p. 295). Another opportunity is afforded by *Chlamydomyxa labyrinthuloides* which when at rest lives as a plant and when active like an animal (Parker and Haswell, '97, p. 49).

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One point more. The close resemblance of mitochondria, amounting almost to identity in both plants and animals, may indicate a common ancestral form possessing mitochondria. It is a general assumption that plants antedate animals, and it seems significant that among the lower plants, and not in the lower animals, we find groups in which mitochondria have not been detected. I have particularly in mind the *Algæ*, for in this interesting group some forms contain mitochondria and others do not (M. and Mme. Moreau, '15, p. 730). The absence of mitochondria is interpreted in various ways. Some think that the large chloroplast takes over their function (Guilliermond, '13, p. 86) and it is possible that the mitochondrial substance may be present in an invisible diffuse condition. There is every reason to believe that the possession of formed mitochondria is an attribute even more primitive than the possession of a fully formed nucleus, which, unlike the mitochondria, has gradually assumed a more specialized character. Yeast plants in which the presence of a nucleus is debated (Macallum, '99, p. 67) unquestionably contain them (Janssens and Helsmortel, '13, p. 452), and there are indications that some bacteria also have mitochondrial material. This is beside the question, however, because the *Saccharomycetes* and *Schizomycetes* are probably degraded forms. We can look upon the ancient line of evolution as passing through forms closely resembling some of the *Algæ* of the present day which are devoid of mitochondria, into similar forms possessing mitochondria and which gradually acquired fully developed nuclei in addition. From these nucleated alga-like organisms containing mitochondria, the higher plants and the whole animal kingdom gradually evolved. They stood at the parting of the ways. The geological record, incomplete as it is, seems to indicate that the algæ are the oldest of plants; those of the present day being the lineal and comparatively unaltered descendants of the most ancient ones. It is surprising that mitochondria should persist without great modification through all the storm and stress of millions of years of evolution during which everything else changed except perhaps the primitive living homogeneous ground substance. As they were in the beginning so they are now, on the crest of both animal and plant

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evolution, their form in *Vaucheria* (Rudolph, '12, Pl. 18, Fig. 9) being equally indistinguishable from that of the oak and of man. This paper shows how much alike they are everywhere. We are tempted to enquire whether the presence of mitochondria in conjunction with a nucleus made evolution possible. This may indeed be true if, as I have already mentioned (p. 223), they are concerned with protoplasmic respiration, which is perhaps the most fundamental of all vital manifestations. Certainly no consideration emphasizes their importance more.

Summary.

This comparison of mitochondria in plant and animal cells brings to light a truly remarkable degree of similarity.

Their *reactions to fixatives, stains and supravital dyes* are almost identical. Similar fixatives preserve them, modify them and destroy them, in like manner, in both. Even the result of experimental errors in the technique is the same. Plant mitochondria react to the janus green test and stain with supravital dyes in substantially the same way as animal mitochondria, though it is certainly more difficult to obtain a good coloration.

Their *distribution* is almost universal. They occur in all plants with the exception of certain *Algæ*, *Bacteria* and *Myxomycetes*, and no animal has yet been discovered which does not contain them. In addition to this, similar and parallel variations occur in their arrangement in the several tissues and even in the individual cells of plants and animals. They must, therefore, be regarded as an integral, perhaps essential, constituent of living matter.

Their *morphology* is identical in plants and in animals. They assume no forms in the one, which are not present in the other. They undergo similar variations in size and shape in different tissues and in different cells in both. If it were possible to view mitochondria dissociated from their environment, it would be impossible to decide whether they came from plant or animal tissues, provided that they did not contain starch, pigment or some other easily recognizable substance, to serve as a clue.

We have every reason to suppose that their chemical *composition* is much the same in both plants and animals, but here our

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knowledge is for the most part supposition and inference, since direct chemical analyses are obviously out of the question. Their composition, as indicated by solubility with respect to acetic acid, heat and other agents, is certainly subject to similar variations in both.

Their *physiology* is obscure; but their wide occurrence in all protoplasm and their general similarity in all places must mean something. It may mean that, in addition to certain specific functions like the production of chlorophyll, they all have a common duty or part to play in some fundamental vital manifestation like protoplasmic respiration.

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BIOLOGICAL BULLETIN

INHERITABLE MODIFICATION OF THE WATER RELATION IN HIBERNATION OF LEPTINOTARSA DECEM-LINEATA.

WILLIAM LAWRENCE TOWER.

A series of experiments in which *Leptinotarsa decem-lineata* was introduced into the environmental complexes of the deserts at Tucson, Ariz., in nine years of experimentation, have given the results described in this paper; and show how the introduction of an organism from one habitat into another—in these experiments from a mesophytic into desert environment—produce alteration of the water relation in ways that are adaptive in direction and inheritable in character.

The specific result of the experiments concerns the development in the organism, during the period of the experiments, of the capacity to hold water within the tissues so that the intense desiccation of the dry seasons—which are passed in hibernation—does not result in death, and the elimination of the introduced population. The change is adaptive; directly in line with the environmental pressure that is incident upon the population, and in tests is shown to be gametic; in crossing with the normal behaving as a Mendelian dominant; and is not easily reversible.

The materials in the entire series have been pure strains from my laboratory at Chicago; their history, reactions, and genetic composition known for generations before introduction into the Arizona deserts. The materials have at no period in their history been subjected to any experimental operations at Chicago, but are the natural animals, reared under normal conditions, under continuous observation and record. Two strains have been used, No. 99 and No. 100, both derived from nature on the

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banks of the Calumet River near Chicago, and therefore characteristic of, and in their activities, adjusted to the conditions of the place of origin.

These stocks have two yearly generations: pass the winter in the adult condition, hibernate in the soil, emerge in the spring, as soon as the temperature is high enough; and begin breeding to produce the first summer generation which matures in July; this first summer generation breeds at once, giving the second summer generation maturing in August or early September. These do not breed, but after feeding undergo preparations for, and enter, hibernation during September or early October, where they remain until the arrival of awakening conditions in the following spring.

In nature this reproductive rhythm is never pure nor regular; many of the overwintering population remain alive, or are late in emergence from hibernation, and are able, therefore, to cross with the first summer generation, giving hybrids between two differing conditions in the rhythm of reproduction, and all materials direct from nature are liable to be in one way or another heterozygous with respect to this rhythm. Pure lines, or stocks in which the rhythm of reproduction was homozygous, have been used in these experiments and no others.

The second summer generation does not mature its gametes, and after feeding undergoes changes preparatory to hibernation that are common to insects; the elimination of waste products and the reduction of the water content of the tissues, lowering the freezing point thereof, so that the animals are not killed by the low temperatures of the winter months, an operation common to many plants and animals in resting conditions. Upon emergence in the spring, or for breeding, this water reduction is rapidly compensated for by water derived from the food and that absorbed by the tissues from the atmopshere or hygroscopic water.

The location chosen for the experiments at the Desert Laboratory of the Carnegie Institution of Washington was on the edge of the broad flood plain of the Santa Cruz River at the foot of Tumamoc Hill, a deposit of gravel and boulder beds with a thick surface layer of adobe soil, of uniform texture and com-

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position. All experiments were in cages six feet square and three feet high, with wooden or cement bases that extended into the ground two and one half feet, or below the depth to which the animals burrowed in hibernation. These cages were covered with sixteen mesh pearl wire cloth, which acted to soften the rigors of the desert environment, especially the temperature, rate of evaporation and wind flow, so that the materials while subjected to the rigors of the desert did not receive the whole intensity thereof, but approximately the average conditions.

Throughout the food has been the cultivated potato, as in the original habitat of the materials. All watering was by irrigation about the roots of the plants, and in all respects care was taken to prevent the experiments from having any other than the desert environment about them.

Caged experiments were used instead of isolated locations in the open for two reasons: first, observation and record, as well as protection, could best be maintained in the location and in the arrangements as devised; and second, in caged experiments all selective action by animals that might use these beetles as food was eliminated and the results would, as fully as possible, be those directly due to the physical environmental complex. It developed in the course of the experiments that the arrangements provided not only eliminated this source of complication, but that predaceous enemies, parasites, and epidemics were also absent, no trace of any of them having been discovered, so that as far as I am able to discover, the results are solely the product of the action of the physical environment upon the introduced animals.

The Experiments.

All materials were introduced at the opening of the summer rains, and taken directly from hibernation to Tucson and introduced into the cages, there giving two generations in rapid succession, the second going into hibernation late in the summer, depending upon the environic condition. The following series have given the data for this paper:

T 99, introduced 1908, now in hibernation F_{18}.

T 100, introduced 1909, carried to F_{12} and lost in making repairs.

T 100 A, introduced 1911, now in hibernation in F_{12}.

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T 100 B, introduced 1912, now in hibernation in F_{10}.
T 100 C, introduced 1915, now in hibernation in F_4.

Each introduction was taken from the stock at Chicago in the following way: On emergence from hibernation the materials were, of each sex, placed in containers, covered with a cloth, and twenty to twenty-five of each sex removed without inspection, so that there would be no opportunity for selective action on my part, and this parental group of forty to fifty individuals were taken or sent at once to Tucson and placed in the cage prepared with food to receive them. They were then allowed to breed as a population. When the progeny of an introduced group, or if at any other time the population was so large that to breed all was impossible, the same impersonal method of deriving the parental group was resorted to, and it seems highly improbable that with these methods, any selective action could have entered into the series of experiments.

Each introduction has had the same method of derivation from the basal stocks at Chicago, and the same treatment under the conditions of experiment at Tucson, grown as populations, treated as populations, and the results are of the population as a whole, and not of individual lines or mutants.

The series has shown several interesting responses to the introduction into the desert environment, but the most interesting one is the development of a capacity to retain water in the tissues of the second summer, or hibernating generation, instead of eliminating it in preparation for hibernation.

The development of this modification of the animal was first discovered in F_6 of T 99, when a population of over four hundred individuals of the second annual generation was sent to Chicago early in September, 1910 (211 ♂ 234 ♀), placed in a cage in the garden at Chicago, in size and construction like those at Tucson, where they soon entered into hibernation, but absolutely failed to survive the Chicago winter, and were completely eliminated, while a culture of the parent stock in a cage six feet away showed only the normal reaction to that particular winter.

The following year, 1911, tests of this condition were made as follows: T 99 in F_8, of the second annual generation, and T 100 in F_2 of the second annual generation were sent to Chicago

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early in September and hibernated in the garden alongside of the original stocks, and a further test was made of T 99 in F_7 in which the first summer generation was crossed as pairs with the normal stock at Chicago, and their F_1 allowed to hibernate in the garden along with the normal and the stocks from Tucson. The results that were derived in the following spring are shown in Table I.

TABLE I.

SHOWING THE RESULTS OF THE TESTING OF THE SURVIVAL DURING HIBERNATION IN THE WINTER AT CHICAGO.

Population Generation, Stock.	Test, Winter of	No.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
T 99, F_8	1911–12	100♂	87♂	0♂	13	87
		100♀	90♀	0♀	10	90
T 100, F_2	1911–12	100♂	86♂	5♂	14	80
		100♀	89♀	7♀	11	82
*C 99, F_{20}	1911–12	100♂	91♂	80♂	9	11
		100♀	89♀	84♀	11	16
C 100, F_{12}	1911–12	100♂	92♂	83♂	8	17
		100♀	87♀	79♀	13	8
F_1 Heterozygotes:						
♂ T 99 × ♀ C 99		347♂	317♂	0♂	8.6	91.4
♀ T 99 × ♂ C 99		386♀	342♀	0♀	11.4	98.6

* Chicago normal stock.

These tests were made in wire tubes twelve inches in diameter and forty-eight inches long, placed close together, so that all had as far as one can determine the same conditions during the winter. The findings in the emergence from hibernation in May and June, 1912, show, first, that the condition of non-survival in the T 99 materials concerns the entire population, and that in T 100, the same change is coming over the stock but did not involve the entire population, 6.5 per cent. surviving. These surviving individuals of T 100 were placed in breeding cages, where most of them died, two females and one male only taking part in reproduction, giving a first summer generation of eleven males and fifteen females, which died without breeding, although given every condition for reproduction.

The F_1 heterozygous population showed complete elimination, or a total dominance of the condition present in the Tucson parent, which was also present in an equal intensity in both

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parents, and equally dominant. On the contrary, the Chicago stocks showed a rather good rate of survival, about the average, and in that the entire test was in a space not over six feet square, with a uniform sandy soil, the result can hardly be attributed to highly localized differences in the winter conditions.

In the summer of 1912 further and more extensive tests were made as follows:

T 99 in F_{10} in the second summer generation to Chicago in September.

T 100 in F_4 in the second summer generation to Chicago in September.

T 100 A in F_2 in the second summer generation to Chicago in September.

The tests for the year comprise the determination of survival in populations that had been at Tucson two, four and ten generations. The results of these tests as determined in the spring of 1913 are given in Table II.

TABLE II.

Population Generation, Stock.	Test, Winter of	No.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
T 99, F_{10}	1912–13	100♂	89♂	0♂	11	89
		100♀	92♀	0♀	8	92
T 100, F_4	1912–13	100♂	87♂	1♂	13	86
		100♀	86♀	3♀	14	83
T 100 A, F_2	1912–13	100♂	91♂	6♂	9	85
		100♀	87♀	7♀	13	80

In T 99 no survivals were found, and the survivals from T 100, although given opportunity to breed, died without issue, and the survivors from T 100 A gave a first summer generation of twenty-six males and thirty-seven females, these a second summer generation of fifty-five males and forty-seven females, of which two males and three females were able to survive hibernation in the following winter, but did not breed after their emergence from hibernation in the spring of 1913.

In the F_1 hybrids in this year (1912), regardless of the direction of the cross, all were eliminated in hibernation, not one appearing in the following spring, again showing the complete dominance of the Tucson trait over the original or normal condition at

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Chicago. In the F_2, however, in both crosses survivals occur, and out of 2,137 hibernated, 443 emerged in the spring of 1913. Two random matings were made from these, of twenty males and twenty females, and large populations grown for the second summer generation and hibernated under the usual conditions, gave in the spring of 1914 the following results:

	Hibernated.		Emerged.	
	Males.	Females.	Males.	Females.
Mating *A*	861	905	672	689
Mating *B*	919	945	792	807

These in all ways were not to be distinguished from the normal stocks, and allowing for the normal elimination incident to hibernation under the conditions provided, which averages in my garden about twenty per cent., the experiences of the experiment suggest that we have in the F_2 popuulation of 1912

	Completely Eliminated. 1 DD. + 2 Dr.	Surviving. 1 rr.	
Observed	1,694	443	
Expected	1,602.75	534.25	if a normal monohybrid.

In that the normal elimination in hibernation of the Chicago stock runs about twenty per cent., the eliminated individuals in this test of F_2 are surely the combined DD and Dr portions of the population, plus the non-distinguishable dead individuals of the (rr) portion of the array.

This method of experimentation and testing has been continued in each year to the date of writing, the results of which show that the condition of non-capacity to survive the winter at Chicago is a gradually increasing product of the populations at Tucson, and that the behavior in crossing of the Tucson characteristic is uniformly that of a Mendelian dominant, the normal Chicago condition appearing in F_2 in numbers that are as close to expectation as could be expected.

Since the discovery of this condition in T 99, all of the introductions of *L. decem-lineata* have been tested in the hibernating generation with respect to their capacity to survive the winter conditions of the habitat from which the stock came. The results of these tests are presented in Tables III., IV., V. and VI.

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Table III.

Showing in Culture T 100, the Complete Failure of Survival in the Populations of This Culture after Six Generations in the Deserts of Tucson.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_6	1911–12	100♂	87♂	0♂	13	87
		100♀	90♀	0♀	10	90
F_8	1912–13	50♂	47♂	0♂	6	94
		50♀	44♀	0♀	12	88
F_{10}	1913–14	25♂	23♂	0♂	8	92
		25♀	22♀	0♀	12	88
F_{12}	1914–15	50♂	44♂	0♂	12	88
		40♀	39♀	0♀	22	78

Table IV.

Showing the Results of the Tests in Culture T 100 A, in which Elimination During Hibernation is Shown to Increase Progressively, Ending in a Condition in which Survival of the Winter at Chicago is no Longer Possible for the Populations in this Culture.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_2	1911–12	100♂	91♀	6♂	9	85
		100♀	87♀	7♀	13	80
F_4	1912–13	100♂	84♂	1♂	16	83
		100♀	87♀	3♀	13	84
F_6	1913–14	50♂	44♂	0♂	12	88
		50♀	45♀	0♀	10	90
F_8	1914–15	100♂	89♂	0♂	11	89
		100♀	93♀	0♀	7	93

Table V.

Showing the Survival Tests made in the Second, Fourth, Sixth and Eighth Generations of the Culture T 100 B, in which the Progressive Increase in the Elimination During Hibernation from the Second to the Eighth Generation, Results in Complete Eliminatiôn in the Last Two Tests Made in this Series.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_2	1912–13	100♂	89♂	5♂	11	84
		100♀	86♀	7♀	14	79
F_4	1913–14	100♂	91♂	2♂	9	89
		100♀	90♀	3♀	10	87
F_6	1914–15	100♂	86♂	0♂	14	86
		100♀	87♀	1♀ died	13	86
F_8	1915–16	50♂	45♂	0♂	10	90
		50♀	42♀	0♀	16	84

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TABLE VI.

SHOWING THE TEST MADE UPON CULTURE T 100 C, INTRODUCED INTO THE TUCSON DESERTS IN JUNE, 1915, WHERE IT REPRODUCED, THE SAMPLE OF THE SECOND SUMMER GENERATION, TAKEN TO CHICAGO, IN SEPTEMBER, AND SUBJECTED TO THE SURVIVAL TEST IN THE WINTER OF 1915–16.

Population Generation.	Test Winter of	Number Taken.	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_2.....	1915–16	50♂	46♂	4♂	8	84
		59♀	44♀	6♀	12	76

These tests shown in the above tables have been made in the wire tubes, placed close together, and having as far as could be determined the same conditions during a given winter. Undoubtedly the testing in tubes favors the culture in the elimination of possible enemies in the soil, and in fact, the survival of the normal stocks in the tubes at Chicago is higher than in large open cages, so that any action of the tube is favorable to survival rather than otherwise.

In all of the introductions precisely the same action is going on, and in all of the tests the same result shows at about the same rate of progression, namely, that the capacity to survive the winter diminishes progressively as the number of generations increases in which the stocks have lived under the conditions of the Arizona deserts. The greatest change comes between introduction and the hibernation of the first year.

At Chicago for some years I have made routine tests of certain of the activities concerned in hibernation, in that this period of passing of the unfavorable portion of the year in their habitat is a season of possible elimination, of possible selective action, therefore, one of possible effectiveness in the evolution of the population. In Table VII. I have shown a series of tests made in the stock C 100 from the eighth to the eighteenth generations, inclusive. These were all made in wire tubes sunk in the ground, under similar conditions of soil, moisture and climatic exposure.

The tests in Table VII. show that in the preparation for hibernation, on the average, eleven per cent., of the tested materials are not able to complete the preparations and do not enter into hibernation. These will be found dead upon the surface of

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Table VII.

Showing in the Original Culture of *L. decem-lineata* No. 100, at Chicago. Tests Made to Determine the Elimination in the Hibernating Population, in Preparation for Hibernation, the Number that Hibernated, and the Percentage Eliminated During Hibernation, with the Percentage of Survivals in the Following Spring.

Stock Culture No. 100, Chicago.

Population Generation.	Test Winter of	Number Taken	Hibernated.	Emerged.	Per Cent. Eliminated.	
					Before H.	During H.
F_8	1910–11	100♂	91♂	83♂	9	8
		100♀	87♀	80♀	13	7
F_{10}	1911–12	100♂	96♂	82♂	4	14
		100♀	83♀	79♀	17	4
F_{12}	1912–13	100♂	94♂	80♂	6	14
		100♀	88♀	77♀	12	11
F_{14}	1913–14	200♂	181♂	160♂	9.5	10.5
		200♀	173♀	164♀	13.5	5
F_{16}	1914–15	100♂	88♂	71♂	12	7
		100♀	90♀	67♀	10	13
F_{18}	1915–16	100♂	83♂	77♂	17	6
		100♀	91♀	74♀	9	15
Totals		700♂	633♂	553♂	av. 9.583+	av. 9.916+
		700♀	612♀	541♀	av. 12.416+	av. 9.166+
		1,400	1,245	1,094	av. 11	av. 9.541+
Total elimination in connection with hibernation, av.					20.54+	

the ground in the tube, but what the cause of the elimination is has not been determined, and may possibly not be possible of determination as the population will not breed until after hibernation and it is therefore impossible to attempt to get a culture of these individuals for further testing, and further there are no indications that have been detected that they are in any way different from the remainder of the tested population. Moreover the sample for testing has always been drawn from the stock culture, in an absolutely impersonal manner and at random so that the elimination may for the present be regarded as a chance elimination in the preparations for hibernation, of about the same value in each hibernating generation, therefore of no probable modifying action upon the population.

From the same table it appears that during hibernation there is on the average an elimination of nine and one half per cent.

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of the tested populations, or a total elimination incident to hibernation of the second summer generation of these animals of about twenty per cent. of the population, about eighty per cent. emerging in the following spring. In nature, however, the elimination is greater, due no doubt to agencies that are eliminated under the conditions of these tests, which completely cut off all the losses that might come from burrowing animals that might use them as food, and probably other agencies as well.

In Table VIII. are given the combined results of the testing of the introduced cultures at Tucson, respecting their ability to survive the hibernating season of their native habitat.

TABLE VIII.

SHOWING THE COMBINED RESULTS OF THE TESTING OF THE CULTURES T 99, T 100, T 100 A, T 100 B, T 100 C, FROM THE SECOND TO AND INCLUSIVE OF THE SIXTEENTH GENERATIONS. IT SHOULD BE NOTED THAT THE PERCENTAGE OF ELIMINATION BEFORE HIBERNATION, REMAINS ABOUT AT THE AVERAGE FOR THE SPECIES AS SHOWN IN TABLE BUT THAT THE ELIMINATION DURING HIBERNATION INCREASES, UNTIL ABOUT THE SIXTH GENERATION, WHEN IT BECOMES TOTAL, AND REMAINS SO TO THE END OF THE SERIES TO DATE.

Population.	No.	Hibernated.	Emerged.	Per Cent. Eliminated. Before Hib.	Per Cent. Eliminated. During Hib.	Per Cent. of Population Surviving.
F_2 T 100 A.... T 100 B.... T 100 C....	250♂ 250♀	226♂ 217♀	15♂ 20♀	9.33+ 12.66+	84.33+ 78.66+	7.5
F_4 T 1100 A.... T 100 B....	200♂ 200♀	175♂ 177♀	3♂ 6♀	12.5 11.5	86 85.5	2.25
F_6 T 100..... T 100 A.... T 100 B....	250♂ 250♀	217♂ 222♀	9♂ 1♀ died	13 11	87 88.66+	.20
F_8 T 99..... T 100..... T 100 A.... T 100 B....	300♂ 300♀	268♂ 270♀	0♂ 0♀	10 11	90 89	0
F_{10} T 99..... T 100.....	125♂ 125♀	112♂ 114♀	0♂ 0♀	9.5 10	90.5 90	0
F_{12} T 99..... T 100.....	200♂ 200♀	177♂ 178♀	0♂ 0♀	12 11	88 89	0
F_{14} T 99.....	100♂ 100♀	89♂ 93♀	0♂ 0♀	11 7	89 93	0
F_{16} T 99.....	100♂ 100♀	86♂ 90♀	0♂ 0♀	14 10	86 90	0

Comparison of the data in Tables VII. and VIII. shows conclusively: First, that the elimination in the preparation for

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hibernation remains at about a constant figure in both the introduced materials at Tucson and in the original materials that have lived continuously at Chicago; second, that the elimination during hibernation takes a great increase in all the second generations tested, from 9.5 per cent. to over 80 per cent., and then in the next following years increases slowly until the sixth, when elimination is complete and continues to be so, no capacity to resist the Chicago winter conditions appearing in any of the Tucson materials in the later generations.

Beyond question, therefore, some change has taken place as a consequence of the introduction of *L. decem-lineata* from the

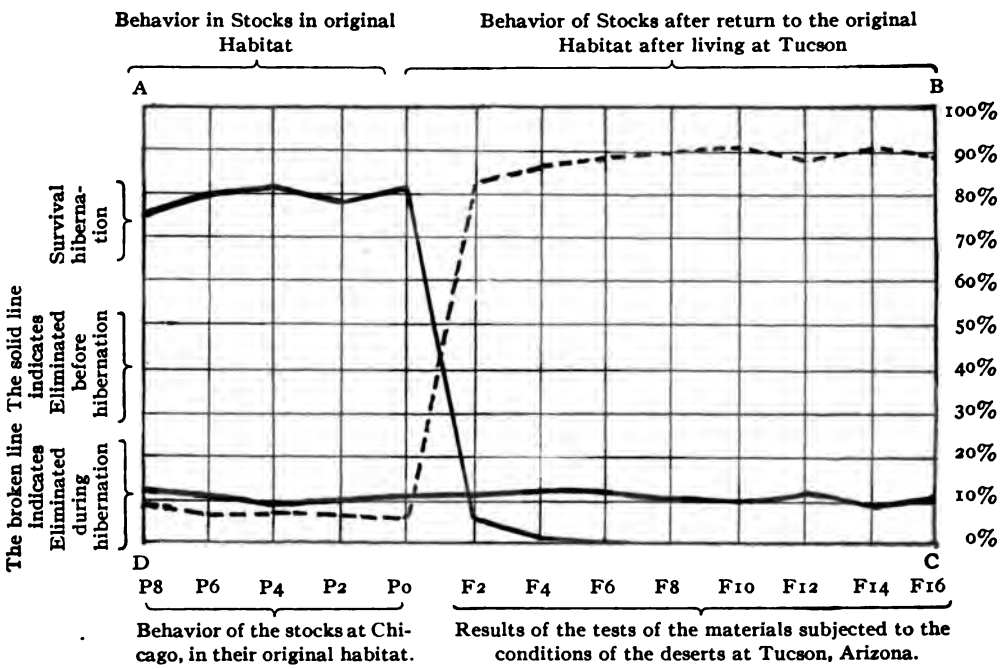

FIG. 1. Graph showing the curve of survivals from hibernation, of elimination in preparation for hibernation, and of elimination during hibernation.

habitat at Chicago into the deserts at Tucson, in the change from a moist habitat into one of intense desiccation during the resting season, which is passed in hibernation. This change is made graphic in Fig. 1, showing the line of elimination in the preparation for hibernation, remaining constant throughout the

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series, the sharp changes in the line of survival of hibernation and in elimination during hibernation that occur between the introduction and the first hibernating period, at the end of the second generation.

In hibernation temperature and humidity in the soil are the two chief factors concerned with these animals, and their action upon the organisms is difficult of isolation. In the two environments chosen for the experiments, the conspicuous difference is one of humidity, not of temperature; soil conditions at Chicago during the period of hibernation having a high and rather constant water content, whereas at Tucson the relation is one of continuously diminishing humidity. At the close of the summer rains at Tucson, the intense desiccation accompanying the high temperatures of the dry after summer, rapidly lowers the water content in the soils until the onset of the winter rains in December, and again in the dry fore summer from the end of the winter rains in March to the beginning of the summer rains in July, the water content is progressively lowered until at the end of the period it may be as low as 5 per cent. or even less in the upper layers of the soil. It is this latter period in which the most intensive elimination of the cultures at Tucson takes place.

In desert organisms, in general, some mechanism is present by which the water content of the resting organisms is conserved during the dry and resting periods in the year, otherwise the plants or animals are not able to survive the hostile conditions of these rigorous portions of the year that are so characteristic of every desert environmental complex, and which may be prolonged, irregular, and highly variable in their manifestation in different years. Consequently, surviving desert types must meet, not only the average extreme and hostile conditions in their existence, but irregular and extreme manifestations of the environic complexes, often of prolonged duration. In other words, a desert environment is not only a most rigorous one, but also about the most variable habitat that an organism has to meet.

The response found in these experiments at Tucson suggests at once that the alteration is one of water relations, one in

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which the capacity to hold the water in the hibernating generation has developed. Tests of the capacity to hold the water in the tissues against desiccation have been made in the different series of experiments, with rather striking results.

These tests are made by taking about equal weights of one of the Tucson cultures from the generation that is hibernating, and from the cultures at Chicago, enclosing them in small

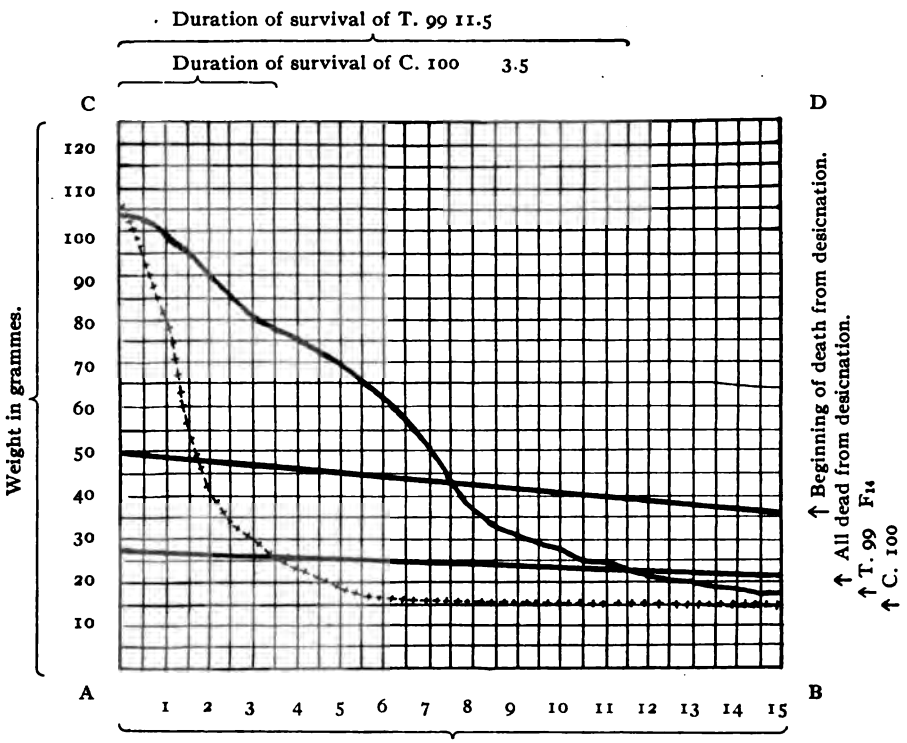

FIG. 2.

wire cloth containers, the two, then subjected to intense desiccation by the passage of a current of dehydrated air at a uniform rate through the materials until death has resulted, and the materials attained a uniform dry weight, weighings being taken every twelve hours.

These tests show that the Tucson materials, especially after

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the sixth generation at Tucson, have constantly the capacity to hold onto the water content in their tissues, are more resistant to desiccation, and in every way are different in their reactions to the water loss than are the same materials that have lived at Chicago continuously. In Fig. 2 I have plotted the results of one of these dual tests made upon T 99 in the fourteenth generation of its existence at Tucson, and the stock C 100 from Chicago.

The curve of response of the materials at Chicago shows a rapid water loss in the first day, the onset of death from desiccation by the end of the second, and the death of all in this test at three and one half days, with the attainment of a constant dry weight under the conditions at about the sixth day. In the sample from Tucson, however, the curve of response to desiccation shows a much less rapid loss, the first deaths not occurring until the seventh day and total death not until the twelfth day, and dry weight constancy about the fifteenth, but not as low as in the culture of Chicago materials. The temperature in this test was constantly 20° C., the temperature used in all such testings.

There is a very obvious difference in the ability of the two sets of organisms to let go of the water in the tissues under-desiccating conditions in the environment. In these tests the conditions were most severe, with absolutely dry and rapid passage of air, in all respects immensely more severe than are ever obtained in any desert complex during hibernation, as the animals are then protected in the ground and desiccation is slow and gradual. However, the desert adjusted group shows that it has the ability to hold the water, or less capacity to let the water loose from its tissues when subjected to the pull of an intensely dry environic condition, and this one difference would decide the fate of either set of materials in different environments.

The eliminations in the survival tests made at Chicago of the samples from the Tucson stocks, are clearly due to their not being able to let go of the water in their tissues with sufficient rapidity to keep the freezing point below the temperature of the soil, consequently they are actually frozen by the decreasing temperatures accompanying the onset of the northern winter, and examination of test cultures made for the purpose at Chicago shows

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that few of the individuals in the test survive beyond December first, and none have been found alive by the end of the same month. Further many of the individuals seen in these examinations when recovered from the ground show frost crystals within the tissues, and extensive disruptive actions thereof.

Repeated tests in the different cultures at Tucson, at early and late generations during their history, show that the retention of water in the tissues against the influences of a strongly desiccating environic complex gives a series of findings that are in close accord with the data derived from the survival tests of the same materials at Chicago; a sharp change in this capacity between introduction and the first period of hibernation, and thereafter a slower increase through the following hibernation periods, until the full capacity is developed, at about the sixth generation after introduction.

From all the different aspects in which this response to the changed environic complex is viewed, it gives only the one conclusion, that there has resulted from the sharp change in the living conditions the development of a capacity to hold water and not lose water in the presence of conditions in the surroundings, which in the original materials causes rapid loss of water with eventual death. The modification is, therefore, of a direct adaptive nature, in direction and kind to meet the needs of the new environment.

What the alteration has actually been in the mechanism of the organism, I do not know. Thus far I have not been able to detect any anatomical modifications, or changes of a cytological character that have taken place. Water loss in these animals is through the dermal glandular secretions and respiratory activity. As far as I can determine, there is no decrease in the dermal glands that are present, either in size or number, or any alteration in their distribution, although I have searched diligently therefor. Moreover, there are no indications of a thicker cell wall, either in the hypodermis or of any increased thickness in the cuticular linings of the tracheal tubes.

Whether it is due to changes in the permeability of the cell membranes or to changes in the colloidal contents of the cells is a matter of opinion, as I have no evidence that supports

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either view with any certainty, and accurate determination of either of these is difficult, if not quite impossible. Regardless of what the actual change may consist of, it is an evolutionary modification, that is directly adaptive in response to altered conditions of life, and as such is well worth careful examination and further investigation.

Perchance it may seem that it is easy to arrive at results of this kind. To prevent any such misapprehension, the experiences in the introduction of other species into the same environic complexes may be cited. During the years in which these experiments with *L. decem-lineata* have been in progress, there have been introduced with the same care and persistency *L. signaticollis*, *diversa*, *undecimlineata*, *panamensis*, *multitæniata*, *oblongata*, *haldemani*, *juncta*, and *dilecta*, representing a diversity of environmental origins and adjustments to temperature and moisture during the hibernation period, and in none of them has anything like the result described in *decem-lineata* been found. The closest approximations thereto are in the species *multitæniata*, *oblongata*, and *signaticollis*, all of which live in haibtats having rather high rates of desiccation during the resting season, and survival in the case of these introductions has been irregular, and the product of favorable conditions in the particular winter, rather than any alteration resulting from the introduction. All of these species, further, live in areas in which the temperature in the resting season is fairly high, so that there is no necessity for the reduction of the water content to prevent elimination by frost action.

Inheritance.

It was shown in connection with some of the earlier tests that the alteration when crossed with the normal condition at Chicago, gave an F_1 that was completely eliminated during the test of survival of the winter, and that F_2 showed a result suggesting the survival of the recessive extractives of the normal type. Further crossings of these have confirmed this original finding and extended the information as to the behavior of the alteration in inheritance, thus showing that the change between the conditions of the populations at Chicago and Tucson is of a gametic nature, and not somatic or temporary.

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All tests of the heritability of this modification have been made with the sixth or later generations, for the reason that the alteration was apparently not at its full development until then, and in that the only means of distinguishing between the two conditions has been the survival test under the winter conditions at Chicago, earlier generations in which there were survivals of this test, would have given only results that had an error in them not possible of estimation. After the sixth generation at Tucson, all the cultures have thus far given the uniform result in crossings with the normal at Chicago, regardless of the direction of the cross, and these summated results are given in Table IX.

TABLE IX.

F_1 Populations.	Pairs.	Population.	Total Tested.	Hibernated.	Emerged.	% Eliminated.		Survived.
						Before.	During.	
Pairs { T. 99 × C. 100; C. 100 × T. 100; T. 99 × C. 99; C. 99 × T. 99 }	42	7	2,987	2,651	0	11.25 ±	88.75 ±	0
F_2 population	65	11	6,411	5,814	1,573	9.3 ±	66.1 ±	24.4 ±

In making these tests of the heritability of the alteration in the Tucson cultures, the F_1 series were all from matings of the first summer generation, whose progeny would hibernate, and for the F_2 tests, the over-wintering generations at Tucson and Chicago were crossed at Chicago, and the second summer generation tested. The results show in all that the F_1 population are in no instance able to survive the test, that the condition of the Tucson cultures is dominant, but that there is a segregation in F_2 and a survival from the test that is for the total series 24.4 per cent., about the expected proportion of the extracted recessives. These in breeding in F_3 show no indication of the Tucson condition, either in pairs or mass cultures, all surviving the hibernation test in F_4 in all respects like the normal Chicago materials.

The behavior in inheritance shows, therefore, no points of interest aside from the fact of its heritability, and the fact that a condition that has arisen rapidly, but apparently progressively, behaves in crossing when fully established as a Mendelian

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dominant, but its behavior in a Mendelian way is no indication of its origin after its production.

Nature of the Alteration.

As already shown, the alteration in the Tucson cultures is physiological, involving a specific aspect of the reproductive cycle, that of the resting period which is passed in hibernation, and the only sure test of its existence is that of the survival under the conditions of the northern winter, at the place of origin of the stocks. This fact limits the possibilities of testing and experiments with the modifications that are possible, since no certain structural alterations in correlation with this change are demonstrated up to the present, and the differences in the color in the materials are too insecure criteria to be used at present.

What the physical or chemical changes are there is no indication at the present time, and one interpretation has about the same chances of being the correct one as any other excepting for the fact that the modified materials show a difficulty or slowness in water loss which seems to me to suggest more probably some altered condition in the colloids.

The chief question that concerns us in the consideration of the nature of the alteration, is whether it is an added or new condition, or the revival of an ancestral one.

The species *L. decem-lineata*, originally confined to the eastern slopes of the Rocky Mountain uplift, and eastward over the Great Plains into western Kansas and Nebraska, from whence it gradually spread eastward to the Atlantic coast, has as its nearest relatives, a series of species that are confined to the high plateaus of Mexico; areas of semi-desert with arid conditions, especially in the resting season. Using the usual criteria of kinship, similarity in structures and habits, the species *L. multitæniata* and *oblongata* are the nearest relations of this northern species phylogenetically.

All three species interbreed rather freely, are in developmental sequences similar, as in their ecological relations, in their reproductive cycles, in relation to the growing season of the habitat in which they live. These relations and conditions I have

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recorded in a previous publication, along with the probable relations and phyletic history and relations of these organisms.

Both of the southern species in all of the locations in which I have information concerning them, must encounter during their period of hibernation desiccation in differing degrees of intensity, from a rather low one on the Plateau of Ahnuac, to intense conditions in the deserts of San Luis Potosi, and in hibernation do not go deep in the soils, but remain in the upper layers, or near the surface, whereas *decem-lineata* in its range goes deep in the soil. Owing to the disturbed and impossible conditions in Mexico, since this condition was discovered in the Tucson cultures, it has been impossible to either obtain new materials from the Mexican locations, or make desirable observations and tests that might have been made had more happy conditions prevailed. Fortunately some experiments made in 1908 and 1909 for other purposes have given data as a by-product that is of interest in this connection.

In both of these years crosses were made in the garden at Chicago of the C 100 stock and freshly obtained materials of both *L. oblongata* and *L. multitæniata*, the latter from Chapultepec near the City of Mexico, the former from Cuernavaca in the State of Morelos, Mexico. These were of the first summer generation in all species, and the F_1 populations were hibernated in the garden at Chicago under the full intensity of the winters of 1908–9 and 1909–10, with the results that are shown in Table X., all the materials being from the mating of pairs, and not from population cultures.

The experiences in the crossing of the three species shown in Table X. with the elimination of the hibernation F_1 population during the winter months is highly suggestive, and would give the basis for an interesting series of tests were it possible to obtain fresh materials from the Mexican locations. The complete dominance of the Mexican species over *decem-lineata* in the matter of survival in hibernation, indicates that in them the same or essentially the same condition exists as in the cultures at Tucson. Unfortunately, I have not had the necessary materials for the testing of their resistance to desiccation, and can therefore only conclude that the close identity in the survivals

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from hibernation is due to the same causes as it is in the tests of the Tucson cultures. Unfortunately, I have no data concerning the survival of the Mexican types at Chicago that are at all comparable, so that I am not sure whether the pure Mexican

TABLE X.

			Pairs.	F_1.		Hibernated.		Emerged.	
L. 10 lin. C. 100	♂ × ♀	L. mult Chap.*	1908–09	7	170♂	196♀	147♂	171♀	0♂	1♀ died
			1909–10	14	278♂	304♀	229♂	258♀	2♂ died	1♀ died
"	♀ × ♂	"	1908–09	6	145♂	144♀	119♂	123♀	0♂	0♀
			1909–10	10	309♂	301♀	267♂	271♀	1♂ died	0♀
					902♂	945♀	762♂	823♀	3♂ died	2♀ died
L. 10 lin. C. 100	♂ × ♀	L. obl. Cuer.†	1908–9	4	96♂	104♀	74♂	91♀	0♂	0♀
			1909–10	6	201♂	197♀	161♂	174♀	0♂	0♀
"	♀ × ♂	"	1908–9	5	131♂	127♀	100♂	93♀	0♂	0♀
			1909–10	9	246♂	265♀	197♂	213♀	0♂	0♀
					674♂	693♀	532♂	571♀	0♂	0♀
L. obl. Cuer.	♂ × ♀	L. mult. Chap.	1908–9	2	77♂	79♀	64♂	60♀	0♂	0♀
			1909–10	7	193♂	201♀	154♂	183♀	0♂	0♀
"	♀ × ♂	"	1908–9	3	66♂	73♀	60♂	64♀	0♂	0♀
			1909–10	4	107♂	119♀	91♂	99♀	0♂	0♀
					443♂	472♀	369♂	406♀	0♂	0♀

* Chapultepec. † Cuernavaca.

types are able to survive the Chicago winter or not, but it is my opinion that they would not.

The two Mexican species introduced into the deserts at Tucson showed in the first introductions in 1908 a huge elimination during hibernation, but they survived in the following year and continued a precarious existence for several years thereafter. Later introductions survived, or failed, for reasons not associated with the behavior in hibernation.

Upon the basis of the probable phylogenetic relations, the facts of the developmental cycles and the similarity in the ecological relations and needs, the data in the crossing of the species under the Chicago conditions and the survival values of the F_1 found therein, and the obvious recessiveness of the northern species in its hibernation behavior to the southern, gives the basis for several conclusions of interest as to the nature of the change.

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(1) Assuming that the phylogenetic picture that we have drawn of the three species is essentially correct, and that *L. decem-lineata* is the younger derivative from the Mexican types, an essentially fair conclusion as far as conclusions in this line are at all possible, then the results of the tests show that there is a dominance of the older phylogenetic condition over the newer; or

(2) It may be concluded that in the origination of *L. decem-lineata* in its northern range, there had been a loss of an agent in the organization, which when present, produced retention and slow loss of water under desiccation, and its absence ease of loss, the experimental tests showing, therefore, the dominance of the presence over the absence. In that the reaction in crossing is a monohybrid one, one unit of difference may be assumed; or

(3) It may be concluded that we are dealing with a physical state in the living mechanism that exists as two conditions, alternative to one another in crossing reactions, but, which by the pressure of the medium may be altered to present first one aspect of its reaction, and under other conditions of the medium, the opposite aspect of its effect in the reactions of the living mechanism.

Which of the three possibilities is chosen as the correct one will depend entirely upon one's philosophical background, and from each a rather entertaining and plausible argument can be made. From purely mechanistic and physico-chemical considerations of living phenomena, I am inclined to the third possibility, as not only the more probable and thinkable, but also the one that as a working hypothesis offers the only opening for further experimental investigation.

In this preference I am no doubt prejudiced in my choice by many experiences with the behavior of the non-hibernating generation in which the behavior is easily, rapidly and positively altered, reversed at will by changes in the water content of the medium, producing reversibility of many of the trophisms of that generation. However, in this first summer generation, another condition of the reproductive cycle is under observation, and in that the reproductive cycle itself is present in two sharply alternative conditions, to breed or not to breed, activity or

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hibernation, and further since these states are known in experiments to be alternative in hereditary behavior, one must not be too fast nor certain in arriving at conclusions in these complicated physiological activities.

Regardless of what one may believe concerning the alteration or the findings of subsequent investigations, certain aspects of its nature are demonstrated, its sharp alternativeness in heredity, its rôle in the determination of survival in different habitats, its directly adaptive nature; and its alteration in direct response to the conditions of the medium in adaptation to those conditions; these aspects of its nature show an alteration, a character capable of alteration, in ways that are of importance in the survival of the organisms, and that might be of decided value in evolutionary activities, speciation, and in ecological and distributional relations and results.

Method of Origin.

This account deals with experiments that are concerned with populations, and the results seem to be those of the alteration of the population as a whole. As shown, the experimental method was such that as far as the operator was aware, the choice of materials was random and entirely impersonal, and throughout any personal influence has been eliminated as far as known. Further, the living of the cultures in cages eliminated the action, if any, of enemies and further gave the cultures better conditions of life than they would have had in nature. Insofar as is known, therefore, the series deals with the response of an introduced population to the physical conditions of its environment. What are the methods of origin of this response and alteration? There are several possibilities:

1. That the original population consists of different pure lines, which under the conditions of the original habitat interbreed, and are hidden from recognition, but that under the Tucson conditions one has been able to survive with the characteristics found, and that the progressive development of its full intensity is the gradual elimination under the conditions of the experiment, of all but the one line with the characteristics finally developed. The conditions of experiment are, therefore, acting only as a sieve to separate out those capable of survival from those not

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capable thereof, and we are determining in the experiment only some aspects of the composition of the population of the species in its original habitat.

2. That the introduced populations are uniform in composition, but that as the result of the altered conditions of life, variations are induced, of which only those whose reactions enable them to resist desiccation have survived; a natural selection of the fit by the physical environic factors.

3. That the population has latent within it the factor for water retention, and that this latency from an ancestral conditions under the conditions of the new habitat, is reactivated, mutates to the ancestral state in the materials of the experiments.

4. That the water relation in connection with hibernation is one due to some physico-chemical relation in the living colloids, that exists in one of two aspects, a condition of the substance, its type of manifestation depending upon the nature of the conditions of the medium.

There may be other possible methods whereby the condition is produced in the experiments, but these seem to me the most obvious possibilities.

That the results are due to the isolation under experiment from the introduced population of a pure line does not seem probable, or at all possible under the conditions of these experiments. All the basal populations introduced came from cultures in which the reproductive cycle was known to be homozygous with respect to the rhythm of the two generations, and since any heterozygousness in this rhythm is at once manifested and very obvious in the cultures, and was also repeatedly tested for in the stock cultures, and none found, the evidence is against the assumption. Further all the introduced stocks were taken from the original stocks on emergence from hibernation, after all the elimination incidental to hibernation had taken place. Were there lines in the original materials with the conditions that later developed in the Tucson cultures, which were isolated by the selective action of the Tucson experiments, and could not later survive the Chicago conditions when returned thereto; why, if present in the original stocks, were they not also eliminated by the conditions of the winter hibernation in the parental habitat?

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Further in the elimination incident to hibernation, that found in preparation for hibernation remains about a constant percentage (about 11) throughout the series at Chicago and Tucson, the difference in the two is in the elimination that takes place during hibernation. The introduced populations at Tucson were all of individuals that had survived the Chicago winter's hibernation, whereas the materials returned to Tucson for testing did not survive the Chicago winter, as shown in the records of the tests.

There is, therefore, no evidence nor reason to interpret the results of the experiments as being due to the isolation at Tucson of pure lines from the introduced population, and every reason, as shown above, why this interpretation is highly improbable. The pure line concept is so facile and insidious in its possibilities for interpretation, that the result of the series of experiments are easily interpreted to conform therewith.

Since 1910 I have tried to develop pure lines from the materials at Chicago with respect to this water retention, and have not up to the present the least evidence that such exist, nor has the slightest success attended my efforts along that line. I cannot go beyond the facts of experience and must, therefore, conclude that upon the basis of the evidence available, pure lines of the characters developed in the Tucson experiments do not exist in the parental materials, and that the results are not due to the sieve-like action of the Tucson environment upon the introduced population, and I believe that this conclusion is entirely justified upon the basis of facts at present available.

It may be assumed that the condition developed was in correlation with some other characteristic operative at breeding, and that introduction eliminated those not possessed of this trait, but in these introductions, as in the T 99 series, from actual observation the entire introduced population took part in the initial breeding, and nowhere have I found the least indication that there has been any such relation, although I have been upon the lookout for them.

That the results are the product of the selection of favorable or adapted variations surviving from among multifarious variations, regardless of size, a product of natural selection, is without

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any confirmation. The chief change takes place between introduction and the first period of hibernation in the elimination during hibernation, and there is therefore no opportunity for the operation of such selective action, even though it is present in the following two or three hibernations. I have tried to devise tests of this point, thus far without any success, and rather extensive breeding of pedigree pairs from these cultures have thus far given only negative results in this line. While I admit its possibility and its value as a basis for argument or interpretation, I am still without any evidences at all that can be used either way concerning this possibility, and am of the opinion that it is not, in view of the findings, a plausible interpretation of the results.

Concerning the possibilities of the reactivation of latent agents, under the conditions of experiment, a plausible interpretation could be made, if the initial assumption is made that such latent agents exist, an assumption that might receive some support from the probable phylogenetic history of these animals. Were any such condition latent in these beetles, it would seem that some indication would have been found of it in the rather extensive experiments that have been in progress in the last ten years, especially those that have to do with the reproductive cycle and hibernation; but as far as I am aware, there has been no indication thereof, and finding of it would have been of considerable value in many of my experiments, and after all the finding of the latency would not in this instance be of much service in the interpretation of the results.

It seems to me that the most plausible interpretation of the results of these experiments is that we are dealing with a case of the response of the entire introduced population, of uniform composition and behavior, in a determinate variation in Darwin's sense. A population reaction to the conditions of a new environment in a characteristic that is capable of existence, and does exist in species now living in alternative conditions depending upon the environic constellation encountered. That the alteration is directly adaptive in its manifestation implies neither purpose nor use, only that in the relation of water in the tissues, mechanistic considerations show at once that only two possible

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conditions can exist; to hold the water or to let go of it, and there are no other possibilities. In other words, the alteration is adaptive and directive in its response, because from the physical nature of the materials no other relations are physically possible.

A crucial question is, why has the reaction not been as easily reversible on return to Chicago conditions? In this lies interesting possibilities for investigations now in progress. Up to the present the reversibility of this action has not certainly been obtained, although I have no doubt but that if I can discover the proper relations between the internal mechansim of these beetles and their medium that the reverse alteration of the Tucson condition to that at Chicago will be found.

Whether the reversal ancestralward, or what may be assumed to be an ancestralward direction, is easier than progressively, is for the present is a matter of opinion, and I am unable to discover any physico-chemical reason why it should be so, but we know many reversible actions that go easily through one series of events in one direction, and with more difficulty in the reverse and through another series of operations. In these purely physical and chemical reactions, ancestral influence is not at all involved, and I see no reason to assume the influence of ancestry and phylogenetic relationship in living reactions of the same general physical type.

Evolutionary Significance.

In the evolution of populations, in their habitat adjustments as in their distributional phenomena, reactions, accommodations of this sort may well have played a by no means minor rôle, and the value of adjustments of the kind found in these introduced populations at Tucson in these operations in nature are at once too obvious to need mention. Physiological adaptations of many sorts may well have their origin and owe their diversification to operations of precisely this general type, the fact of their heritability or behavior Mendelianwise having no significance or indication of the method of origin. In fact this and other experiences with these animals through many years strongly inclines me to the conclusion, that Mendelian alternativeness is

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no certain criterion that they arose discontinuously. Osborn has presented from the findings of the fossil record, certain facts concerning the phylogeny of characters in mammals that now behave Mendelianwise in crosses, but, which arose, as far as the data of paleontology can decide, by gradual and continuous modification, and not by mutative jumps, and further in that the Mendelian reactions concern only extremes or alternatives in the same quality of the organism, it may result that the entire Mendelian phenomena are but the expressions of characters whose manifestation is capable of easy and reversible manifestation.

The fact that this alteration is inheritable at all, may to some, seem incomprehensible, but it never segregates, its behavior in heredity is always that of a mono-hybrid, and in all experiences it behaves as a property characteristic of the whole, and possibly involves, therefore, an alteration not of determiners in the usual sense, but an alteration of the colloidal matrix of the tissues, soma and germ, which we must regard not as different kinds of substance, but as different expressions of one and the same specific kind of living material.

From this series of experiments, operations that are important in population evolution, especially of introduced populations, are certainly discovered, and has served in my hands for an entering wedge into the experimental investigation of questions concerning the evolution of populations, an aspect of evolution of which there is extremely little experimental analysis or investigation. In nature and the diverse products of the evolution phenomena in nature, our classification, schemes of phylogeny, our experiences are with populations of diverse things, in associated relations, both living and non-living, and more intimate knowledge of, and a broader understanding of these population problems seems most necessary for further progress in evolution investigations.

As an example of the manner, and the change necessary, and its production in the alteration of an organism originally characteristic of a mesophytic habitat into one in all ways adjusted to the rigors of the Arizona deserts, of the nicety of adjustment, its promptness, and direct adaptive nature, these experiments

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provide illuminating examples and the basis for continued investigation of the same general aspect of the evolution problems.

DESERT BOTANICAL LABORATORY,
CARNEGIE INSTITUTION,
ZOÖLOGICAL LABORATORY,
UNIVERSITY OF CHICAGO,
April 17, 1917.

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ON THE CAPACITY FOR FERTILIZATION AFTER THE INITIATION OF DEVELOPMENT.[1]

CARL R. MOORE.

I. An Attempt to Fertilize Sea-urchin Eggs Subsequent to Hypertonic Parthenogenesis.

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I. Introduction.

The purpose of the present paper is to present a part of the results of a study of the capacity for fertilization of eggs of the sea-urchin (*Arbacia punctulata*) after the initiation of development by artificial agents. A previous paper[2] by the writer has dealt with some of the phenomena encountered in an attempt to fertilize the eggs of *Arbacia* after a treatment with certain artificial stimuli, *e. g.*, butyric acid and heat. The ideas involved resolve themselves into the question—can eggs that have been activated by artificial parthenogenetic agents, be fertilized by spermatozoa of the same species; or does activation by hypertonic sea-water preclude fertilization as in the case of sperm activation?

[1] From the Marine Biological Laboratory, Wood's Hole, Mass., and the Hull Zoölogical Laboratory, University of Chicago.

[2] Moore, '16.

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The egg of the sea-urchin cannot be fertilized before maturation has taken place, even though the spermatozoön may enter the egg. Penetration is not fertilization. However, if a spermatozoön enters immediately after maturation, normal changes characteristic of fertilization are produced. But shortly after the act of fertilization the egg returns again to a condition in which fertilization is impossible. E. B. Wilson[1] has demonstrated for *Cerebratulus* that if pieces of fertilized eggs are dropped into sea-water containing active spermatozoa there is no reaction between the pieces of eggs and sperm; however, with pieces of unfertilized eggs there is a reaction. Delage, too, has noted that starfish eggs which had been exposed to artificial parthenogenetic agents could not be fertilized.[2]

Maturation, then, leads to changes within the egg that make possible initiation of development by either a spermatozoön or an artificial stimulus. And if this initiation of development is brought about, carrying with it of course a change of the egg system, fertilization is thereby rendered impossible.

It would seem strange not to suppose that an egg passes through essentially similar physiological changes during the development from one cell to a swimming pluteus, whether activation has been produced artificially or by a spermatozoön. If activation by a spermatozoön produces a physiological state of reactivity that leads to development, and, if activation by artificial agents also produces physiological changes that lead to the same kind of development, should we not suppose that the egg—the only common factor—would pass through essentially similar conditions in the two cases? One would judge from the papers of Loeb, that this supposition is erroneous, that sperm will react with artificially activated eggs in an entirely normal manner, producing normal swimming larvæ, while it is positively known that such is not the case when activation has been previously affected by means of a spermatozoön.

The writer has presented proof that eggs of the sea-urchin activated by butyric acid, and resulting in the production of normal membranes, will not react with sperm nor become fer-

[1] Wilson, '03.
[2] Delage, '01.

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tilized, as Loeb has believed,[1] but that they are entirely unresponsive to sperm, even though spermatozoa pass into the egg substance. The present paper deals with an attempted analysis of the conditions that are produced, after activation by hypertonic sea-water, with reference to the capacity of the egg for fertilization.

Loeb[2] determined that an exposure to hypertonic sea-water (50 c.c. sea-water + 8 c.c. 2½ m. NaCl) for a certain period of time would cause sea-urchin eggs, after their return to normal sea-water, to segment in quite a normal manner: they would also undergo further developmental changes and become essentially normal plutei. But certain eggs from the culture will not have had a sufficiently optimum exposure to bring out their full developmental tendencies and will as a result of this remain quiescent after dividing into two, four or eight cells Can these eggs activated by hypertonic sea-water yet be fertilized by a spermatozoön? Loeb says they may be and uses the assertion as a proof that the egg has not been changed in its power of normal physiological reactivity; and that it may be fertilized if only a spermatozoön reaches the cortex of the egg. His hypothetical "lysin-like" substance contributed by the spermatozoön can still assert itself by producing cytolysis and his "corrective agent" of the spermatozoön may also act to check the imposed cytolytic condition and allow the egg to go on to development.

If the conditions are as Loeb believes, we will naturally have to assume that activation of the egg—the initiation of development—is accompanied in the two instances: (1) in sperm activation and (2) in activation by artificial parthenogenesis, by a different series of reactions that lead to the same end, namely the production of swimming larvæ. But if this is so we will have to assume a lack of harmony in the development of an egg when activated by different agents. In the vast number of cases in which development may be artificially initiated there is no common factor but the egg itself. Development is a series of reactions on the part of the egg whether initiation is given by a

[1] Loeb, '13.

[2] "Art. Par. and Fert.," pp. 237–238.

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spermatozoön or by an artificial agent and, unless we assume such a relatively restricted set of reactions, we have as yet no explanation to offer for parthenogenesis. The principal issue at hand is, can an egg of *Arbacia* that has been once activated by hypertonic sea-water be fertilized or reactivated by a spermatozoön of the same species?

The results obtained from this study show conclusively that a treatment of the sea-urchin egg with hypertonic sea-water for an optimum length of time—*e. g.*, that in which one obtains the highest percentage of development, both in regard to cleavage and to larval production—leads to changes in the physiological state of the egg that result in the loss of its capacity for fertilization. Spermatozoa readily enter the eggs but they are unable to cause a reactivation of the egg; they appear only as inert bodies within the cytoplasm. Not only does the spermatozoön fail to produce activation changes but also the initial changes, produced by the hypertonic treatment, have created an environment in which a spermatozoön very quickly goes to pieces.

If an egg has not received the optimum exposure for activation, from the hypertonic treatment, a spermatozoön is capable to some extent of provoking activation changes, and a very striking gradient of activity on the part of the egg can be demonstrated from the normal behavior in fertilization to a complete state of responsive inactivity. If the previous treatment has led to segmentation, spermatozoa readily enter the blastomeres, but are entirely incapable of reactivating them.

And finally, the capacity for fertilization has been found to correspond with the presence or absence of the egg secretion, fertilizin. If the egg possesses any capacity for fertilization after the hypertonic treatment, fertilizin has been found to be present; when the capacity for fertilization is negative, no fertilizin has ever been detected by its sperm agglutinating properties.

II. Material and Methods.

The material used in this investigation has been confined to the Atlantic sea-urchin (*Arbacia punctulata*) found in the Woods Hole region. Fresh material was received daily from the labor-

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atory live-car and was kept in running sea-water in the laboratory until used. Eggs and sperm were secured in the usual way—by cutting away the leathery oral disk and collecting the eggs and sperm in separate syracuse dishes as it is shed by the animal. Eggs were always washed in sea-water from two to four times before being used in an experiment, as this insures a more consistent lot of fertilizations than with unwashed eggs.

The method for testing the capacity for fertilization of eggs that have been activated by a hypertonic solution is the same as that employed by Loeb[1] in his studies of this nature. Eggs after washing were transferred to the hypertonic sea-water and allowed to remain for definite periods of time: at the termination of the exposure they were removed with a pipette to two separate dishes of sea-water (150 c.c.–200 c.c.) A and B. Lot A was allowed to stand at room temperature without further treatment, while to lot B was added a quantity of a fresh sperm suspension. For each experiment both a fertilized and an unfertilized lot were set aside as controls.

III. Observations of Living Material.

Since there is a great difference in the effects of different concentrations of, and variable time exposures to, hypertonic sea-water the experiments with the sea-urchin egg have included quite a range of concentrations and periods of exposure, but to simplify the data, only results from two concentrations of the hypertonic solution will be reported at this time. The two concentrations may then be known as the weaker solution (50 c.c. sea-water + 8 c.c. 2½ m. NaCl) and the stronger solution (50 c.c. sea-water + 16 c.c. 2½ m. NaCl).

These two concentrations of hypertonic sea-water were employed because of the very different results obtained with them. An exposure to the weaker solution results in the segmentation of a relatively few eggs (see Table I. and Fig. 1, *a*) but usually a very small percentage of the eggs will segment once or twice and cease their development, while a similar exposure to the stronger solution gives a much higher percentage of cleavages and a much greater number of swimming larvæ. An exposure

[1] "Art. Par. and Fert.," pp. 235–236.

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to the latter solution is much more nearly an optimum treatment for activation than is the weaker concentration.

1. *Fertilization after Exposure to Hypertonic Sea-water.*

The fundamental interest of the writer has been directed towards the capacity of an egg, once activated by an artificial stimulus, for fertilization. If an egg is fertilized normally, the spermatozoön supplies the optimum stimulus for the initiation of changes, latent within it, that lead to normal development. But since these same changes may be initiated (it seems that we must regard it so) by artificial agents, is there yet the possibility of an observable stimulus from a spermatozoön if applied subsequent to or during these changes? Can fertilization be superimposed upon hypertonic parthenogenesis?

Eggs were exposed to the action of hypertonic sea-water as given above, returned to normal sea-water and divided into two lots, one of which remained standing at room temperature, while to the other was added a fresh sperm suspension. Let it again be brought to mind that exceedingly variable results are obtained from the same type of treatment of two different lots of eggs and since the condition of the eggs before sperm are added is the all-important factor for subsequent fertilization one must be very careful to know as thoroughly as possible the condition of the eggs in question. In one experiment one may obtain only 3 per cent. of cleavages after a given hypertonic treatment and at another time, under as nearly as possible identical conditions, 25 per cent. to 35 per cent. cleavages. The following experiment will indicate the method of procedure and the results in general that have been obtained.

From the results of this experiment one can readily see the different capacities for fertilization possessed by eggs exposed to the weaker solution (lot A) and those activated by the stronger one (lot B). This relation can be seen at a glance from Fig. 1, the curves of which were plotted from this experiment.

The lot of eggs returned to normal sea-water and inseminated, after an exposure to the weaker solution A, for a period of ten to twenty minutes, appear very little, if any, different from the control lot of normally inseminated eggs, either as regards

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Experiment:

4:30 P.M. Eggs collected and washed. Aug. 26, 1916.

5:00 P.M. Divided into two lots *A* and *B*.

A, placed in (50 c.c. sea-water + 8 c.c. 2½ m. NaCl).

B, placed in (50 c.c. sea-water + 16 c.c. 2½ m. NaCl).

Eggs were returned to sea-water from the two solutions by means of a pipette and divided into two lots one of which remained without further treatment (control): to the other was added immediately a fresh sperm suspension.

TABLE I.

Lot A.

No.	Returned to Sea-water.	Hypertonic Solution Alone. Percentages of		Hypertonic Solution Plus Sperm. Percentages of	
		Cleavages.	Swimming Larvæ.	Cleavages.	Swimming Larvæ.
1	5:10 P.M.	0	0	99	90
2	5:20 "	0	0	95	85
3	5:35 "	1	0	83	70
4	5:50 "	2	0	70	55
5	6:00 "	3	1	60	35
6	6:20 "	2	0	28	20
7	6:35 "	2	2	33	20
8	6:50 "	4	0	30	15
9	7:05 "	5	1	28	5
10	7:20 "	4	1	20	15
11	7:35 "	6	0	35	10
12	7:50 "	8	0	35	5
		Lot B.			
1	5:05 P.M.	1	0	22	25
2	5:10 "	0	0	18	18
3	5:20 "	10	7	12	20
4	5:30 "	22	20	20	28
5	5:40 "	18	7	18	5
6	5:50 "	14	5	9	2
7	6:00 "	12	1	10	1
8	6:20 "	5	0	1	0
9	6:35 "	6	0	6	0
10	6:50 "	4	0	1	0
11	7:05 "	2	0	2	0

the number of cleavages or the number of swimming larvæ. But since an exposure of this intensity is practically ineffective in producing initiation of development, is it not to be supposed that the eggs can yet be fertilized by sperm? They are, so far as we may judge, entirely normal or essentially so. However, as the length of exposure to the hypertonic solution is prolonged the capacity for fertilization drops off until after an exposure of two hours' duration scarcely any larvæ appear as a result

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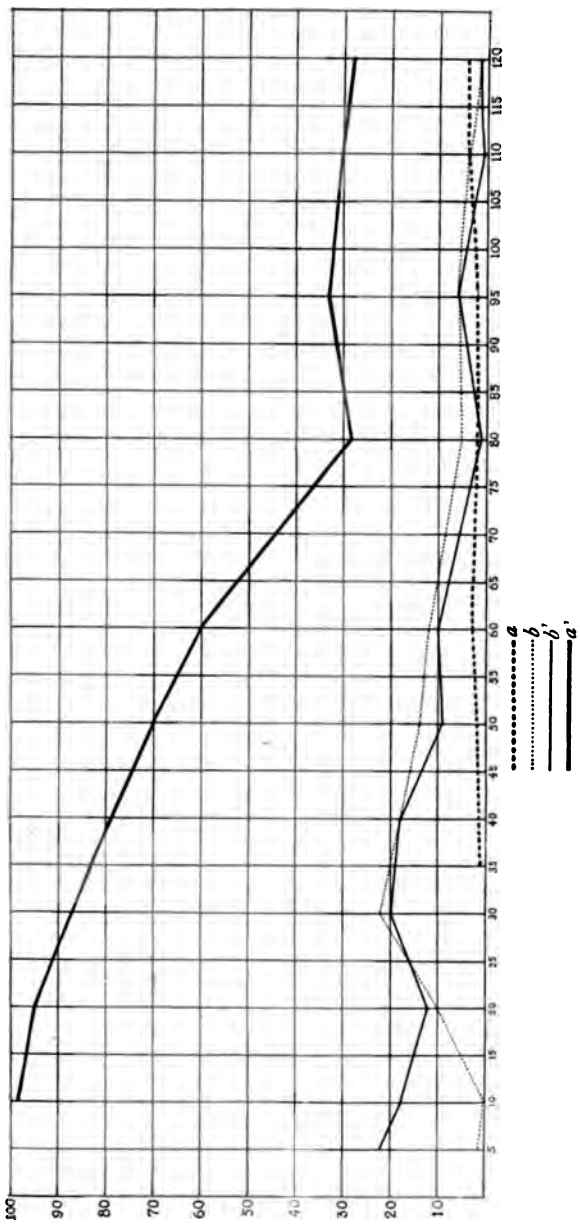

FIG. 1. Curves showing the initiation of development of *Arbacia* eggs as measured by cleavages, after an exposure to hypertonic sea-water; and the relative capacity for fertilization after an exposure to the different concentrations. *a*, weaker hypertonic solution alone; *a'*, weaker hypertonic solution followed by insemination; *b*, stronger hypertonic solution alone; *b'*, stronger hypertonic solution followed by insemination. The ordinates represent percentages of cleavage; and the abscissæ, time of exposure in minutes.

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of insemination.[1] This is undoubtedly due to the condition of the egg, produced as a result of the hypertonic exposure· its capacity for fertilization is being gradually decreased.

We may correlate, it seems, this gradually progressing unfertilizable condition of the egg that appears as the eggs are allowed to remain in the solution, and the escape of substances from the eggs which also increases as the eggs are allowed to remain for longer times in the solution. One can readily notice an almost entire absence of pigment from many of these eggs while others will show a decided loss of it. Probably also many other substances escape at the same time from the egg, some of which may be absolutely necessary for certain chemical combinations taking place during fertilization and development.

The type of swimming larvæ obtained in such a series of experiments shows also a very beautiful gradient in normality as well as in number. Most of those obtained as a result of fertilization, after a short hypertonic treatment, appear entirely normal, but the percentage of abnormalities increases as the length of exposure to the hypertonic solution before fertilization was lengthened: after an exposure of 1½ hours to 2 hours practically no normal larvæ are obtained and the mortality is very high.

For the higher concentration the curve (*b* and b^1, Fig. 1) is decidedly different from (*a* and a^1), the weaker one, but yet it is quite characteristic for exposures to such a concentration. The greatest percentage of cleavages from the hypertonic solution alone are obtained in lots, the exposure of which had been thirty minutes or forty minutes in duration while often a small amount of segmentation is induced by an exposure of 1½ hours. But distinctly different from the curve of cleavages when the lower concentration was used is the fact that the curve of fertilization (?) follows more nearly that of the hypertonic treatment alone. This is especially true when the percentage of swimming larvæ is considered. In dishes of inseminated eggs, previously exposed to the stronger hypertonic solution for 10,

[1] The number of cleavages after a two-hour exposure in this experiment is exceptionally high as compared with scores of other experiments for the same length of time. Usually such an exposure is followed by not more than 3 per cent. of swimming larvæ but many times swimming forms do not appear at all.

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20, 30 or 40 minutes, quite a goodly percentage of larval forms are to be seen, but compared with the curve of larval production of the hypertonic solution alone it has been increased very little or not at all. We cannot, therefore, refer the swimming larvæ appearing in these inseminated cultures to fertilized eggs but they can be regarded only as larvæ that have developed from the action of the hypertonic solution upon the eggs. Insemination did not increase the number of cleavages or swimming larvæ over that obtained after hypertonic activation alone.

We can now consider more in detail the results of the experiment under consideration and let it serve to illustrate the results of most of the experiments.

In an exposure to the weaker concentration of hypertonic sea-water very few eggs give evidence of a cortical change that in any way resembles the fertilization membrane produced around the egg when normally fertilized, and in keeping with this, very few of the eggs segment. It is difficult to say whether or not every egg that cleaved had previously undergone a cortical change. It is difficult even to say if an egg has cleaved or not, for the cleavage patterns have been so variously modified, are so irregular and these are scattered promiscuously among so many eggs that are undergoing degeneration that it is very difficult to determine whether an egg has segmented or is slowly falling to pieces. Many times the cytoplasm of the egg pulls apart, leaving a wedge-shaped opening that finally separates the egg into two approximately equal halves with the nucleus contained entirely within one half; and yet at other times nuclear division may occur without cytoplasmic division. But that there is not a membrane produced that will hold the blastomeres together as in normal fertilization is very evident from the fact that even the first two cells may fall apart and become entirely separate from each other or be held together only within the clear transparent jelly layer of the egg—the chorion layer. But a small percentage of the eggs do produce slight membrane changes: the membrane appears around the egg and can be easily seen with the higher powers of the microscope. They are slightly thickened, clear jelly-like membranes adhering very close to the egg and are not at all to be confused with a normal

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membrane. It is therefore possible to assume that all eggs that have segmented have been preceded by cortical changes of some intensity at least.

As one may see from Table I., the largest percentage of cleavages obtained from an exposure to the weaker hypertonic solution (Lot A) was 8 per cent. and that the greatest number of swimming larvæ was 2 per cent., therefore many of the eggs had segmented that did not continue their development to the swimming stage. The optimum effect of the solution for initiation of development was not imparted to the eggs and development ceased after a few cleavages. In the great majority of eggs, however, no evidence of initiatory changes are discernible and one would naturally suspect that they were yet capable of fertilization, and this, as the experiment shows, is partially true. The shorter exposure eggs when inseminated immediately after return to sea-water, react quite normally with sperm, producing a fertilization membrane, normal cleavage and a high percentage of swimming larvæ. But as the exposures are lengthened there is a gradual decline of the normal conditions until an exposure to hypertonic solution, sufficiently prolonged, will entirely prohibit fertilization of the eggs.[1] After an exposure, lasting 25 to 30 minutes, to this concentration, the eggs begin to show a tendency for loss of pigment: they are lighter in color and a few are so affected as to be reduced to mere shadows of their former condition, but this is much more true of the longer exposures.

The character of cleavage and of larval development also declines from the normal as the curve of fertilization approaches zero. In the experiment cited, dishes A 1 and A 2 gave practically normal cleavage and 85 per cent. to 95 per cent. of swimming forms that appeared practically normal. Beginning with dish A 3 however and extending throughout the series of exposures, cleavage became less and less normal; the cleavage planes had grown more indistinct and in many eggs division of the nucleus had gone on while the cytoplasm remained unsegmented. Some of the dishes presented such an indistinct lot of cleavages that it was almost impossible to judge whether or not an egg

[1] Lack of fertilization after exposure to solutions of various salts, has been pointed out by R. S. Lillie, '11.

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had cleaved but the cleavage count is as nearly correct as very careful examination could make it. The larvæ appearing in dish A 1, after insemination, were a good lot: approximately 70 per cent. swam at the top of the water and more than 50 per cent. are entirely normal. Compared with the swimming forms of A 5, obtained after fertilization, there is a great difference: only a small percentage of the eggs subjected to the hypertonic solution for one hour (A 5) and inseminated immediately, gave rise to swimming larvæ; and of these only a few could be seen swimming at the top of the water. Almost any form of moving masses of protoplasm may be found among the forms of this dish from a very few apparently normal forms to masses that gave only the least bit of quivering movements; blunt masses, angular forms possessing no semblance of morphological similarity to a normal form, dumb-bell shapes one half of which swims and pulls the other lifeless half around with it, bits of spinning protoplasm that appear somewhat as half, quarter or eighth blastulæ are to be found abundantly. As the length of exposure to hypertonic solution is increased before insemination one encounters a more and more abnormal lot of larvæ; there is not only an increase in the degree of abnormality but the percentage of swimmers is gradually reduced until dish A 11 (inseminated) contained only 10 per cent. of eggs that gave the least signs of life, and of these not any swam at the surface of the water; A 12 (inseminated) revealed not a single form that approached the normal.

It is well to point out again the inability of one to obtain an absolutely correct larval count, for among the eggs that have not been fertilized are many that have undergone cytolysis; the count is only relatively correct but it will be sufficient to illustrate the decrease of the capacity for fertilization of eggs that have been exposed to the activating influence of the weaker solution of hypertonic sea-water.

When the more concentrated solution is used as an activator and is followed by insemination, a different condition is encountered. After the hypertonic treatment alone, of say thirty minutes, one obtains a much larger percentage of cleavage and a decidedly higher percentage of swimming larvæ than is

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obtained after the same exposure to the weaker solution. But a comparatively short exposure (5 to 10 min.) to the stronger concentration results in the production of quite a considerable number of membranes. The membranes too are decidedly more pronounced than those produced after exposure to the weaker solution but are not yet like the fertilization membrane produced in normal fertilization, even though the physiological processes responsible for their production may be analogous except perhaps in intensity. Cleavage, after this exposure, although departing very greatly from the normal, is not characterized, as are many of them after the weaker solution, by the separation of the blastomeres; the cells remain in close proximity to each other—a fact that may possibly be associated with the more rigid membrane that is produced around the egg—and a larger percentage of them reach the swimming stage than of those from the other solution. But here also as the length of exposure is increased and the curve of cleavage falls, the same decline in degree of normality and of percentage of swimming larvæ is apparent.

In keeping with the more pronounced effect of the stronger solution in the production of membranes is the fact that the eggs suffer a greater increase in permeability. Loss of pigment is more pronounced than in the case of the weaker solution as one would expect. A small percentage of swimming larvæ obtained after a relatively short exposure to the stronger solution are somewhat normal, but the lots grade so gradually into abnormality and immobility that many can be seen to possess but very little power of movement. Many half, quarter or eighth sized larvæ are found here, as well as following an exposure to the weaker concentration.

There is revealed here a general shake-up of development that is quite characteristic, not merely for the hypertonic treatment, but for development after treatment with any agent that tends to destroy the delicate physico-chemical balance of an egg.

This experiment which may be considered a typical one, offers no indication that the addition of a sperm suspension to eggs that have been activated by hypertonic sea-water are yet

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capable of being fertilized, but on the contrary many eggs that have failed to respond outwardly to the activating influences, by segmenting, are found to be incapable of fertilization. The reaction differences of the eggs or their different physiological states assert themselves by the variety of reactions given by the egg after insemination; one egg will segment and produce a swimming larva; another will divide a few times but finally disintegrate; yet still another will remain to all appearances in a normal condition, being normal in size and color, will be surrounded with spermatozoön and yet does not divide; while finally one will retain its full rounded appearance but give up practically all its pigment content to the surrounding water, but does not segment. If the eggs are given the optimum exposure for cleavages,[1] insemination does not increase either the percentage of swimming larvæ or of cleavages.

Professor Loeb, however, makes the claim that sea-urchin eggs may undergo activation, from an exposure to hypertonic sea-water, to such an extent that they segment once or twice, as the case may be, but are nevertheless still capable of fertilization. Quoting from Loeb:[2]

"When we put the unfertilized eggs of *Strongylocentrotus purpuratus* directly into hypertonic sea-water . . . and if we put them back at different intervals into normal sea-water, we find that if eggs have been exposed a sufficiently long time (two hours or more) to the hypertonic sea-water a number will begin to segment. These eggs will often go into the two- or four-cell stage, or sometimes to the eight- or sixteen-cell stage, and then stop developing . . . such eggs remain after this perfectly normal and they have the appearance of small unfertilized eggs. If we wait for sometime, say twenty-four hours, to make sure that they neither develop nor disintegrate, and add sperm, each one of these blastomeres forms a tightly fitting membrane. They begin to develop in a perfectly normal way and into normal larvæ."

Whether or not the fundamental principles of development in the egg of *Arbacia* differ from those of *S. purpuratus*, the

[1] In this particular experiment an exposure to the stronger solution for 30 minutes.

[2] "Art. Par. and Fert., p. 237.

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results obtained by the writer with the eggs of *Arbacia* do not agree with the observations of Professor Loeb on the eggs of the California sea-urchin.

The eggs of *Arbacia* will, as does the above cited instance of behavior of the *Stronglyocentrotus* egg, segment into two or four cells and go no farther in development, but remain for several hours apparently normal half or quarter eggs. This condition is perhaps best obtained in *Arbacia* following a hypertonic treatment of thirty to forty minutes, with the weaker solution. If such a lot of eggs are returned to normal sea-water and allowed to remain at room temperature (21°–23° C.) for twenty-four hours, many of them will appear quite normal, while all gradations of loss of pigment can be found. There are present also many small eggs of half or quarter normal size but the majority of these are not normal; most of them are entirely or partially devoid of pigment; some few of them however do appear normal in color. If sperm are added to such dishes and the effects of the insemination carefully noted, a few very striking facts are to be observed:

(1) The smaller eggs as well as the eggs that have not segmented are surrounded by actively swimming spermatozoa but they do not produce a fertilization membrane;[1] (2) many of the unsegmented eggs will segment, but the blastomeres are not held together. Since a good membrane is not produced, the blastomeres may become entirely separated from each other or be retained within the very thin jelly layer of the egg and not be in contact with each other as they are normally. These blastomeres may again divide giving rise, each, to two daughter blastomeres that may again become separate, making four individual blastomeres from the same egg or four quarter-eggs; and these may go on dividing until a great mass of cells are present but not held together. Some few of them (for instance all those derived from one of the first two daughter cells) may remain in loose approximation and perhaps give rise to a very

[1] In many cases eggs that have been exposed to the weaker concentration for a short time only, will produce a quite typical membrane after insemination, but those exposed for one hour to this solution, and allowed to remain standing for twenty-four hours, are almost entirely devoid of any semblance of membrane production after insemination.

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abnormal and much smaller swimming larva than the normal. On the other hand, the writer has watched for long periods of time the half- or quarter-eggs found in the dish at the time of insemination but has not found for certain a single one of these that produced a membrane or has segmented as a result of fertilization. But if one should only casually observe the eggs following such a treatment there is no way to distinguish between the separated blastomeres of an egg that has been fertilized and has segmented into two blastomeres that have become separate, and one of the half- or quarter-eggs that was present at the time of insemination. Of importance also is the fact that the *character of a swimming larva of reduced size found in these inseminated cultures is no criterion that would indicate from what kind of an egg it was derived.* As before stated, swimming larvæ of all sizes and of most any external shape assumed by protoplasm can be found in dishes, the eggs of which have been exposed to the hypertonic treatment alone. How then may we conclude that a half- or quarter-sized larva obtained in such a mixed culture after insemination, was derived from a half or quarter-sized egg by fertilization? The only possible way in which the writer could know the exact conditions of the egg from which the swimming larvæ came was by the very laborious method of isolation of individual eggs, and this method was adopted as a last resort.

Four or five hours after an exposure to the weaker concentration of hypertonic sea-water one often finds that 2 per cent. or 3 per cent. of the eggs have segmented once, producing two equal blastomeres that are separated from each other but remain enclosed by the thin transparent jelly layer of the egg. By means of a very fine capillary pipette connected with a flexible rubber tube held in the mouth, these two separate cells, or half-eggs, were isolated from the cultures while in focus under a binocular microscope and collected into a small dish. Thus it was definitely known that only blastomeres of one kind were present. These isolated half-eggs could then be observed and be inseminated in a pure culture, and hundreds of them were isolated and studied in this manner, enabling the writer to determine the following points:

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(*a*) Not a single blastomere of the first cleavage of an egg (a half-egg) isolated and left standing in normal sea-water at room temperature, appeared entirely normal at the end of twenty-four hours.

(*b*) A few of these blastomeres, without further treatment, segmented again and some many times. This cleavage was often delayed some hours after treatment, indicating that developmental processes were going on very slowly.

(*c*) Not a single normal larva was obtained out of hundreds of these isolated blastomeres inseminated seven hours or longer after the treatment.

(*d*) These inseminated, apparently normal blastomeres did not form a fertilization membrane.

(*e*) Out of the hundreds of half-eggs that have been inseminated less than 10 per cent. have ever segmented. It is just possible that some of these cleavages have been due to activation by spermatozoa, but the eggs had been so weakened that cleavage was abnormal and never reached the swimming stage. *But beyond doubt most of these cleavages were due to the slow development going on within the egg similar to* (*b*) *above.*

With these facts in mind is it not possible to account for the results of Loeb without considering that his half- or quarter-larvæ came from the fertilization of the separated blastomeres of the 2- and 4-celled stage? As pointed out above, eggs that have remained standing for 24 hours after hypertonic exposure do not produce membranes after insemination, that are rigid enough to hold the blastomeres together; and in these experiments, as well as in Loeb's, many dwarf and badly deformed larvæ can be seen in the culture but they do not come from the fertilization of a half- or quarter-egg, but rather from an egg that was fertilized and later lost cells. Numbers of swimming forms can be seen trailing a comparatively huge mass of cells after it, that have been lost from the egg itself and consequently has resulted in a reduction in size. Even in the cultures producing swimming larvæ from the effects of the hypertonic solution alone, one encounters these half-, quarter-sized, and even smaller swimming masses representing parts of eggs, but these too are reduced in size from loss of cells during the division of a

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whole egg and possibly also from an egg, one half of which may have become separated after the first cleavage.

The fact that among these 24-hour cultures of eggs, insemination is not usually followed by a perceptible membrane, may lead one to consider the second division of an egg as a division of two half-eggs that have segmented since insemination but not as a result of it. If these first two blastomeres from the cleavage of an egg are separated and each divides again, how is one to judge whether this is the real condition or that perhaps they may represent the fertilization of a half-egg? Decidedly not by periodical examination, but only by continual observation, if in a mixed culture, or by isolation; and as has just been pointed out, hundreds of isolated blastomeres have been inseminated in pure culture but not a single swimming form has been obtained from these, that have remained at room temperature seven hours between the time of hypertonic treatment and insemination.[1] Whether this explanation will serve to harmonize the differences of opinion or whether there is an individual variation between the fundamental processes of development in the two different species of sea-urchin eggs, the writer is unable to say.

But to obtain a further insight to the observable effects of the addition of sperm to eggs that have been caused to start their developmental processes, eggs from a large number of experiments have been preserved and sectioned and the cytological results are presented briefly in a following section.

2. *Fertilizin and Fertilization.*

In a former paper the writer considered in some detail the relation of the presence of the sperm agglutinating substance, fertilizin, liberated from the normal eggs of arbacia into sea-water in which they have been standing, and the capacity of eggs for fertilization, both normal eggs and eggs that had been treated with various reagents. It is not the purpose of the

[1] Some time must be allowed before insemination because of the fact that some of these blastomeres continue to divide before insemination; even seven hours does not entirely eliminate this source of confusion, for a blastomere may divide after that length of time. Developmental changes are still going on, but at a very slow rate.

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present paper to take up for discussion the disputed points of the fertilizin theory[1] nor to discuss the way in which fertilizin acts or its rôle in the process of fertilization, but it is perhaps desirable to add another instance of the existing parallelism between the presence of a detectable amount of this agglutinating substance and the capacity for fertilization. Professor Lillie and the writer have previously shown this parallelism to exist in all cases thus far tested: (1) Normal, ripe *Arbacia* eggs liberate this substance into the sea-water as long as they are capable of fertilization; eggs whose germinal vesicles have not broken down do not liberate fertilizin in detectable quantities and they cannot be fertilized. (2) If *Arbacia* eggs have been exposed to the optimum concentration of butyric acid and full membranes were produced, no fertilizin was detectable and the eggs could not be fertilized. (3) If eggs are over-exposed to the same concentration of butyric acid (1 to 3 mins.) the eggs can be, at least, partially fertilized and fertilizin is readily detected. (4) If they are exposed for two hours to this same concentration of butyric acid (50 c.c. sea-water + 2.8 c.c. N/10 butyric acid) they cannot be fertilized and fertilizin is not liberated in detectable quantities. (5) If eggs are exposed to heated sea-water of 35° C. for 10 minutes they could not be fertilized nor could fertilizin be detected; and finally to be added from these experiments. (6) If eggs are exposed to a hypertonic sea-water solution for one to one and one-half hours, sometimes some of the eggs are still fertilizable to a limited extent but always such conditions have been accompanied by a detectable amount of fertilizin; but in conditions where the eggs are not capable of fertilization no fertilizin has ever been detected. Only one experiment will be given in detail to show this parallelism (see Table II.).

From microscopical examination at 5:00 P.M., August 15, one could see that many eggs of the X lot were apparently normal in appearance; they were well rounded, and seemed to possess practically the normal amount of pigment, but upon insemination they did not produce fertilization membranes and the blastomeres after segmentation were not held together, and not one of them developed to the swimming blastula stage.

[1] See Lillie, '14, Moore, '16.

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Experiment: Aug. 14, 1916.

2:30 P.M. Eggs collected and washed—divided into lot X and lot Y.

3:05 P.M. X put into (50 c.c. sea-water + 8 c.c., 2½ m. NaCl). Y put into (50 c.c. sea-water + 16 c.c., 2½ m. NaCl).

4:25 P.M. Both lots returned to sea-water and lightly shaken to free from jelly, and series of washings begun.

TABLE II.

Time.	Designation.	Lot X.	Lot Y.
4:50 P.M.	Washing 1	$\frac{10}{1.0}$[1]	$\frac{10}{1.0}$
5:10 "	" 2	"	"
5:50 "	" 3	"	"
6:00 "	Inseminated	Cleavage 10 per cent.	0
Aug. 15			
8:30 A.M.	Washing 4	$\frac{10}{1.0}$	$\frac{10}{1.0}$
9:10 "	" 5	"	"
10:45 "	" 6	"	"
11:10 "	" 7	"	"
12:30 P.M.	" 8	"	"
2:10 "	Tested for fertilizin	8 second reaction	negative
	Inseminated	Cleavage 13 per cent.	0
2:15 "	Washing 9	$\frac{10}{1.0}$	$\frac{10}{1.0}$
3:00 "	" 10	"	"
3:20 "	" 11	"	"
4:00 "	Test for fertilizin	5 second reaction	negative
4:20 "	Washing 12	$\frac{5.0}{1.0}$	$\frac{5.0}{1.0}$
5:00 "	Tested	4 second reaction	negative
	Heavy insemination	Cleavage 20 per cent. (poor)[2]	0
5:20 "	Eggs killed in Boveri's picro-acetic acid		

The results of this experiment have been confirmed by a number of others and the results have always been consistent—if the supernatant fluid possessed enough fertilizin to cause the agglutination of a fresh sperm suspension, always there had been eggs present in the culture that possessed enough latent developmental capacity to react with a spermatozoön and to segment as a result of this union, but if no fertilizin could be detected never have the eggs been found to possess a capacity for fertilization. Some change in the physiological or physico-

[1] In every case the numerator of the fraction represents the volume of eggs and sea-water while the denominator represents the volume of eggs and sea-water (in c.c.) remaining in the tube after most of the supernatant sea-water had been drawn off—for further details see footnote 1 on page 276.

[2] No larvæ produced from 5 P.M. insemination, in either lot X or lot Y.

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chemical state of the egg has been brought about as a result of the hypertonic treatment and fertilization is impossible. Of what this change consists we do not know, but it is at least accompanied by an increased permeability of the egg and loss of substances from it. Whether a specific substance necessary for fertilization has diffused from the egg or whether it has undergone new chemical combinations that have rendered it unavailable and whether it may be the substance called fertilizin are questions that cannot now be answered. But it seems significant that here as in all previously modified conditions there has always been a substance escaping from the egg into the surrounding sea-water that could be detected by its sperm agglutinating properties, if the eggs were still capable of fertilization. That this unfertilizable condition is due to a new physiological state of the egg itself and not to a physical condition of the surface of the egg that might prevent sperm entrance, is made certain by observation on preserved material. Sperm do penetrate these eggs and sometimes produce weak changes similar to fertilization or they remain entirely ineffective, depending upon the condition of the egg itself.

3. *Conclusions of Section III.*

The results of this series of experiments seem to indicate that a certain optimum exposure of the eggs of *Arbacia* to hypertonic sea-water initiates changes within the egg that result in development. That all do not segment is because the optimum exposure for one egg is not the optimum for all.[1] Some have been over-exposed, some under-exposed, but if the exposure was the optimum one, swimming larvæ were produced; if less or greater than the optimum perhaps only one or two cleavages resulted and development ceased; if decidedly over-exposed or under-exposed, cleavage does not occur. This quantitative relation is then interesting from the standpoint of fertilization. If the eggs were not perceptibly affected by the exposure they could be fertilized; if the reactions initiated by the exposure were of sufficient intensity to disturb the physico-chemical condition of

[1] The difference may be due to the factor of aging as Goldfarb ('16) has applied the term.

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the egg and cause the loss of substances necessary for fertilization or has caused new chemical combinations to be formed that renders certain substances unavailable, then fertilization is likewise impossible.

In order to determine definitely whether or not this unfertilizable character of the egg is due to a real physiological or physico-chemical condition of the egg substances or whether it is due to physical conditions that prevent the extrance of a spermatozoön into an egg, a large number of these from different kinds of experiments were preserved and sectioned. Usually Boveri's picro-acetic acid was used as a killer and sections (4 μ thick) were stained in iron hæmatoxylin.

IV. Observations from Preserved Material.

The cytological data to be presented is not meant to be a critical analysis of chromosome behavior but is given only to acquaint us with a few of the more general facts of the behavior of sperm in relation to these unfertilizable conditions, and it will be confined to observations upon three different classes of experiments: (1) To mass cultures of eggs that have been exposed to the weaker concentration of hypertonic sea-water for different lengths of time, returned to normal sea-water and immediately inseminated; (2) to mass cultures which have been exposed to the stronger concentration of sea-water for different lengths of time, returned to sea-water and inseminated; (3) to pure cultures of eggs that have segmented but once after an exposure to the weaker concentration, and inseminated.

1. *Insemination Following an Exposure to the Weaker Hypertonic Solution.*

Experiment:

2 P.M. Eggs were collected, washed three times.

2:50 P.M. Transferred to hypertonic sea-water—(50 c.c. sea-water + 8 c.c., 2½ m. NaCl).

Eggs returned at intervals to sea-water, divided into lots A and Ax. Lot A no further treatment; control.

Lot Ax, immediately inseminated after return to sea-water. Eggs from both lots killed as given in Table III.

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TABLE III.

No.	Lot A.			Lot Ax.			
	Returned to Sea-water.	Per Cent. Cleavages.	Per Cent. Swimming Larvæ.	Per Cent. Cleavages.	Per Cent. Larvæ.	Insem-inated.	Killed.
1	3:10 P.M.	2	1	85	75	3:15 P.M.	3:45 P.M.
2	3:30 "	2	0	75	75	3:35 "	4:05 "
3	3:50 "	15	1	45	15	3:55 "	4:25 "
4	4:10 "	25	3	30	8	4:15 "	4:45 "
5	4:30 "	12	0	12	1	4:35 "	5:05 "
6	4:55 "	20	1	12	2	4:55 "	5:25 "

Microscopical Observations on Lot A.

3:30 P.M., A^1 eggs appear normal.

3:55 P.M., A^2 eggs appear normal.

4:15 P.M., A^3 eggs appear normal.

4:40 P.M., A^4 10 per cent. eggs show loss of pigment.

5:10 P.M., A^5 few eggs almost colorless; 30 per cent. appear normal.

5:20 P.M., A^6 some eggs reduced to shadows, many appear quite normal.

Sections of eggs of the AX^1 lot appear not greatly different from lots that have been normally fertilized; very few of them

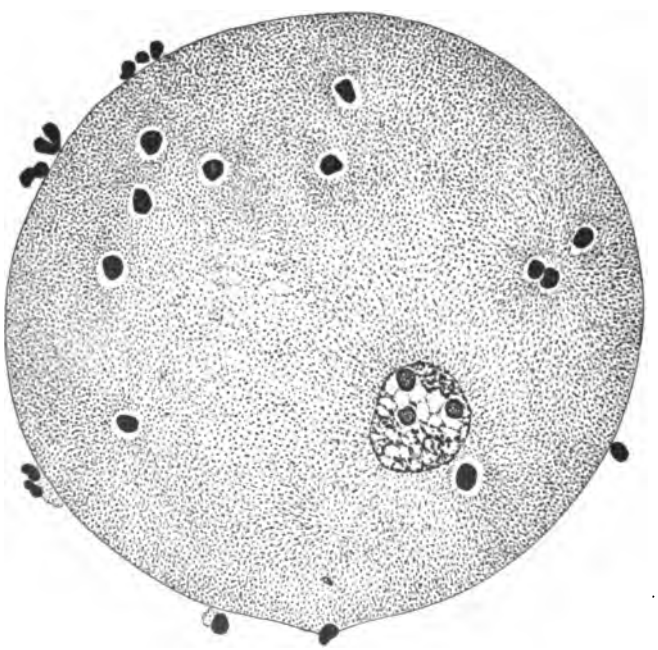

FIG. 2. × 3,000. Section of an egg exposed to the stronger hypertonic solution for 15 minutes and inseminated immediately on return to sea-water. Killed 15 minutes after insemination. The sperm heads are entirely unchanged and have not reacted with the egg.

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show more than one sperm nucleus and this has usually undergone the characteristic swelling and vacuolization changes of a male pronucleus and is accompanied by an aster. After a longer exposure to this concentration of hypertonic sea-water the resemblance to sections of normal fertilization gradually disappears and is superseded by a general condition of polyspermy and later by a non-reactive state of the egg substances,

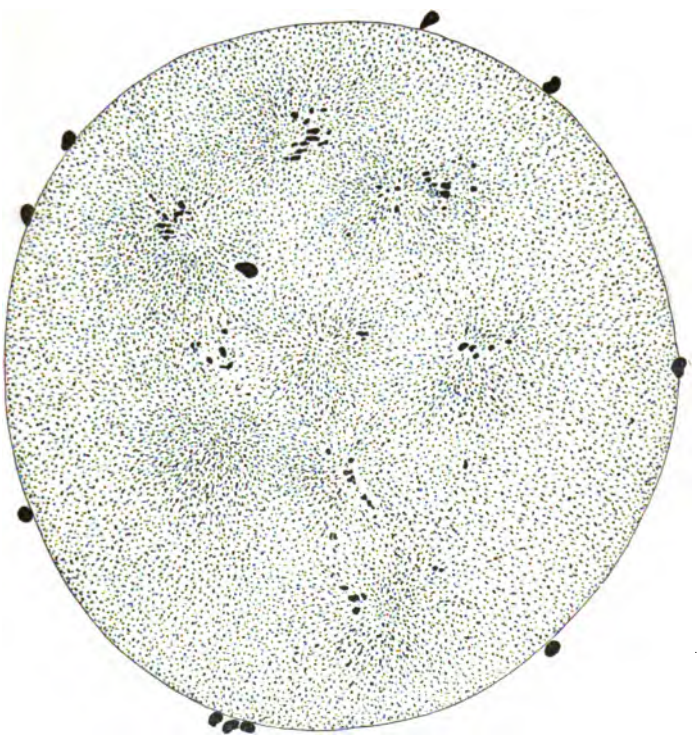

FIG. 3. × 3,000. Section of an egg exposed to the weaker hypertonic solution for two hours and inseminated immediately on return to sea-water. Killed 20 minutes after insemination. All but one of the sperm heads are undergoing fragmentation. The egg nucleus does not appear in this section.

with an abundance of spermatozoa, lying within the cytoplasm, entirely unchanged. Of the eggs of AX^3, probably the larger percentage are polyspermic and show a reactive condition of the sperm heads; they appear swollen and slightly vacuolated and are usually accompanied by an aster more or less well formed. Some sections of this lot still appear almost normal in their

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general characteristics. In lot AX^5 however some of the sperm heads are slightly swollen but are not associated with astral radiations and still others are surrounded with weak radiations. Many cases are encountered in which the egg nucleus has broken down into chromosomes, dispersed along the rays of a unipolar spindle, formed by the egg, as a result of the hypertonic treatment; and at the same time several sperm nuclei may lie unchanged within the cytoplasm of the egg.[1] In AX^6 most of the eggs have almost completely lost their capacity to react with a spermatozoön. The majority of the sections show the sperm heads entirely lacking an aster, but many asters produced by the effects of the hypertonic solution are present and are very definite. The reaction between the egg and sperm is largely negative. In numerous sections the physiological changes within the egg cytoplasm have not only prevented changes characteristic of normal fertilization, but they are even of such a character as to cause the disintegration of sperm heads. Fig. 3 is a section of such an egg and shows one sperm head yet unchanged but several others are undergoing dissolution. Other sections show another type of disintegration which is characterized by a swollen vesicle about the sperm head which is breaking apart and stains very faintly with iron-hæmatoxylin.

2. *Insemination Subsequent to an Exposure to the Stronger Solutions.*

The examination of sections of eggs of the following experiment serves to give a general understanding of the incapacity for fertilization, after eggs have been exposed to the stronger solution of hypertonic sea-water.

Experiment:

4:30 P.M. Eggs collected, washed three times.

5:10 P.M. Transferred to hypertonic sea-water—(50 c.c. sea-water + 16 c.c., 2½ m. NaCl).

Eggs transferred at intervals to sea-water and divided into two lots X and Y. Lot X remains standing without further treatment. Lot Y inseminated immediately after return to sea-water. Eggs were removed from each lot at definite times and preserved as given in Table IV.

[1] See Wilson, '01, and Hindle, '10.

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TABLE IV.

No.	Returned to Sea-water	Lot X.		Lot Y.	
		Percentage of		Percentage of	
		Cleavages	Swimming Larvæ.	Cleavages.	Swimming Larvæ.
I	5:15 P.M.	10	0	25	3
II	5:20 "	6	0	12	7
III	5:25 "	5	2	12	3
IV	6:00 "	20	2	22	2

Series Preserved in Picro-acetic Acid.

I x killed 5:30 P.M.	I y^1 killed 5:30 P.M.	I y^2 killed 5:45 P.M.
II x " 5:50 "	II y^1 " 5:35 "	II y^2 " 5:50 "
III x " 5:25 "	III y^1 " 5:40 "	III y^2 " 5:55 "
IV x " 6:15 "	IV y^1 " 6:15 "	IV y^2

The general cytological observations on this preserved series are as follows: In all lots of eggs exposed to the stronger concentration, from five minutes to fifty minutes, and inseminated immediately upon their return to sea-water, sperm have penetrated the eggs freely and lie in any plane of the egg cytoplasm from the periphery to the center. No attempt has been made to determine by actual count, from sections, the relative proportion of eggs containing spermatozoa, but this condition is found under every field of the microscope and many sections of eggs show from two to sometimes twenty sperm heads. By far the larger number of eggs show the presence of sperm heads imbedded within the cytoplasm.

The effects of the egg cytoplasm environment upon the individual spermatozoön varies in the different eggs in which they are found. Many sections prove by their presence that three, four or more spermatozoa have entered the egg and react with it, thus giving the picture of a typical case of polyspermy; the sperm heads may be separated from the egg nucleus and accompanied by a sperm aster, or two or three may have copulated with the egg nucleus. In other sections a dozen spermatozoa may be scattered throughout the cytoplasm, but thirty minutes after insemination they exhibit not the slightest perceptible change in size or shape (see Fig. 2). They appear perfectly solid with no indications of vacuolization; there is no indication of a sperm aster, and in short the sperm heads appear only as inert foreign

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bodies in the cytoplasm of the egg.[1] Still other sections exhibit a different type of reaction of the sperm to its new environment, that appears significant—the sperm heads may undergo swelling and vacuolization without any indication of the appearance of a sperm aster. For the most part asters arise only in connection with a spermatozoön that shortly appears to be in the swollen, rounded, and slightly vacuolated condition but, as has been mentioned, some possess much more pronounced astral rays than others. This condition of the entire absence of a sperm-aster from the spermatozoön undergoing the more or less characteristic changes of activity, probably to be referred to chemical influences exerted upon it by some substance within the egg, seems to indicate a quantitative reaction of the spermatozoön with the egg, the sperm aster formation being the more sensitive factor. In fact this series of preparations exhibits a gradient of the effects of a spermatozoön, from essentially its normal effect, to a condition in which it provokes not the slightest change; and since a sperm aster is usually associated with a swollen sperm head cannot this be an indication of the suppression of sperm aster formation but a continuation of the more stable changes accompanying sperm activity?

(*a*) *Penetration of the Spermatozoön.*—An interesting condition for the observation of sperm entrance is presented by this series of preparations and since the writer knows of no observations on sperm entrance in *Arbacia* some passing mention of it may be of interest. As may be seen from Fig. 4, two spermatozoa are in the act of entering an egg and many other such sections are to be found among the series. The egg has produced a small protrusion of protoplasm as an entrance cone that projects slightly above the egg surface; and within this cone lies the sperm head pulled out into a narrow chromatin band, one end of which projects down into the cytoplasm. No definitely formed fixation body, to which the inner end of the spermatozoön is attached, has been observed but the very shape and character of the entering spermatozoön would indicate a decided attraction from within the egg that acts upon the sperm head.

[1] Normal sperm asters appear within five to ten minutes. Around most of the sperm heads is a small clear area produced by the retreat of protoplasmic granules of the egg from its periphery.

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Fig. 4 was made from lot III Y[1] (page 283) and perhaps cannot be considered as representing the normal mode of entrance of the spermatozoön of *Arbacia* but it at least indicates the possible method of entrance. It may be necessary to attribute the appearance of the phenomenon to the general retarded condition of the egg protoplasm brought on as a result of the hypertonic treatment before insemination, but it would not seem impossible to discover a somewhat similar condition of sperm

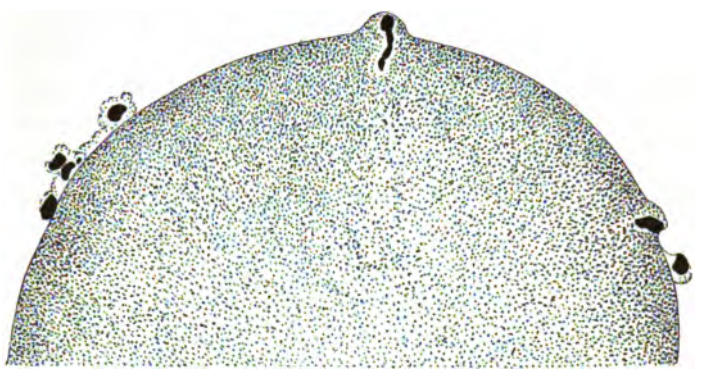

FIG. 4. × 3,000. Part of a section of an egg showing the entrance of the spermatozoön; one has practically completed its entrance and is rounding up, the other in the process of entrance. From same lot as Fig. 2.

activity under normal conditions if the right stages were preserved in a close enough series. Penetration, in *Arbacia*, is effected much more readily than in *Nereis*, in which Professor Lillie[1] has illustrated so clearly the entrance of the spermatozoön. The most essential difference in the morphology of the process in the two forms is the absence of the attraction cone in *Arbacia* which in *Nereis* forms an attachment for the spermatozoön and apparently pulls the spermatozoön through a small aperture in the vitelline membrane as it recedes from the periphery of the egg. Immediately after the spermatozoön is taken inside the egg it assumes its normal appearance, for no elongated heads have been observed that were not in the act of entering the egg.

3. *Insemination of Pure Cultures of Isolated Blastomeres.*

Since the experimental results of the writer did not agree with the views of Loeb—namely, that eggs whose development

[1] See Lillie, F. R., '11 and '12.

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had been initiated by an exposure to hypertonic sea-water, could yet be fertilized, produce fertilization membranes and develop into swimming larvæ, he was anxious to know if fertilization was prevented by failure of the sperm to enter or whether the individual blastomeres—the products of the first cleavage of an egg—had developed an immunity to the latent effects of a spermatozoön.

It is obvious that to determine this point from cytological preparations a pure culture of the first two blastomeres was highly desirable; and with considerable difficulty some two hundred and fifty or three hundred eggs that had segmented but once were isolated from lots of eggs that had been subjected to the weaker hypertonic solution. This pure culture of approxi-

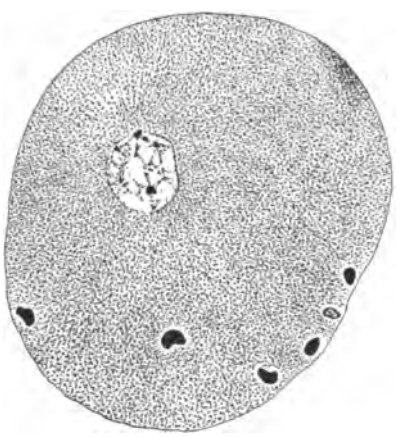

FIG. 5. × 3,000. Section of a quarter egg showing the presence of sperm nuclei. Weaker hypertonic solution, 30 to 40 minutes; egg segmented once, isolated, each blastomere segmented again. Killed 20 minutes after insemination.

mately five hundred half-eggs was heavily inseminated, allowed to stand for twenty minutes and preserved in Boveri's picro-acetic acid. A very few of these blastomeres had divided again into two daughter blastomeres (quarter-eggs) before the lot was inseminated, but every egg was isolated from the mass culture into a pure culture while in the two-celled stage.[1]

Microscopical sections of these half-eggs prove to us beyond

[1] As a definite membrane was not produced by the egg following the hypertonic treatment the blastomeres became entirely separated from each other in the pure culture. All were half-eggs when isolated.

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any possible doubt that sperm do enter the blastomeres (Fig. 5) but the normal effect of a spermatozoön entering an egg is not apparent. Many of the sperm heads lie within the cytoplasm entirely unchanged, while others are seen to have increased in size from swelling, but most of these are undergoing evident degeneration, of which various stages can be seen (Fig. 6). The chromatin gradually loses its staining reaction, appearing much clearer than the unchanged sperm heads outside of the eggs, and some have so entirely lost their staining capacity that they appear only as clear vacuoles scattered throughout the cytoplasm of the half-eggs. Out of the vast numbers of sperm heads observed within these half-eggs only one was found that contained an indication of a good sperm aster. A few of the

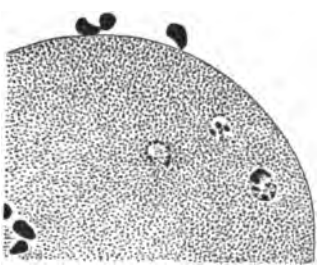

FIG. 6. × 3,000. Part of section of a quarter egg showing presence of both unchanged and degenerating sperm nuclei. No indication of fertilization changes. Same exposure as Fig. 5.

blastomeres are undergoing segmentation as a result of the hypertonic treatment and the spindles appear quite normal.

Here then the spermatozoön not only does not find a congenial environment in which normal reactions can go on, but the surroundings are even hostile and result in the disintegration of the spermatozoön without its having undergone any changes that indicate the least possibility of a fertilization reaction.

4. *Conclusions of Section IV.*

This cytological examination very clearly reveals the fact that the unfertilizable condition of eggs subsequent to an exposure to hypertonic sea-water is not due to the failure of a spermatozoön to enter the egg but that it is due to a change within the egg itself. Certain changes that are to be associated

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with initiation of development have produced an environment in which the reaction between a spermatozoön and an egg has been rendered impossible. *Spermatozoa enter the eggs in comparatively large numbers but the eggs are not fertilized.*

In the shorter exposure to hypertonic sea-water many eggs do react with a spermatozoön but since these are conditions in which a very small percentage of eggs segment and a still smaller percentage reach the swimming stage after the hypertonic treatment, the observations are interpreted as indicating an effect of the hypertonic solution below the optimum exposure for the initiation of development and as a consequence the eggs have experienced but little change from the normal condition; the exposure did not lead to the initiation of development, therefore the eggs still possess their capacity for fertilization. That this lack of a capacity for fertilization is one that is gradually developed as the exposure to the hypertonic solution is increased, is one of the factors that indicates the quantitative side of fertilization. After a short exposure, polyspermy, in which all sperm seem to have a part, is the general rule; a more pronounced effect of the hypertonic sea-water results in a greater number of sperm entering the egg, some showing very weak asters, others being only slightly swollen, while yet a more decided influence may create an environment within which the spermatozoön is not only inactive but also it may even undergo disintegration. Whatever changes have resulted from treatment with hypertonic sea-water, spermatozoa that gain entrance to eggs that have been exposed to optimum conditions for segmentation, or that have been decidedly over-exposed, do not meet with an environment that will permit of a reactivation of the egg.

This degeneration of spermatozoa in a non-reactive egg environment calls to mind essentially similar conditions, encountered normally, in the fertilization of meroblastic eggs such as the pigeon egg described by Blount ('07). Polyspermy in meroblastic eggs appears to be normal but the accessory spermatozoa, after the environment has been changed by reactions within the egg set up by a reaction of the egg with the successful spermatozoön, are repelled peripherally and degenerate and disappear.

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V. Discussion.

For an egg to develop, some stimulus must initiate certain changes within it which, having been once set in motion, appear to be autonomous. If an egg is not fertilized, or its development initiated by some artificial agent, it normally does not develop. But studies in the field of the artificial initiation of development have brought to light many methods that may be employed successfully to set in motion the developmental mechanics latent within the egg. As yet we are unable to determine just the mechanism by which these agents are effective, but practically all methods agree in one essential at least—the permeability of the egg membrane is increased.

Practically every one is agreed that the egg must be considered, a very delicately adjusted physico-chemical system, within which, if a spermatozoön gains entrance, a characteristic series of reactions are set in motion that lead to development and a consequent change of the system. If artificial agents are employed as a substitute for the spermatozoön not only do we have conditions changed but also if the proper procedure is known and applied the end results are essentially the same as when initiation was effected by a spermatozoön, *e. g.*, cleavage, gastrulation and swimming larvæ. After the initiatory changes have been induced a spermatozoön in either case is entirely unable to produce any of its characteristic effects.

The delicately adjusted condition of the egg is only a temporary thing for if sea-urchin eggs are allowed to stand in sea-water for a day or more they gradually lose their power of fertilization. Not only can this unfertilizable condition, accompanied by increased permeability, be produced by standing but also by hypertonic sea-water, butyric acid, by a slight rise in temperature of the sea-water and several other agents including, of course, fertilization.

Many writers have considered an unfertilizable condition as an indication of the death of the egg but to cite only one instance to disprove this, the writer has called attention to the fact that if initiation of development is partially accomplished by a treatment with butyric acid and the membranes so formed are destroyed by shaking, sperm will enter the eggs of *Arbacia* but do

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not assert any capacity for the initiation of development: however if the eggs were subsequently exposed to the proper treatment with hypertonic sea-water a goodly per cent. will develop to swimming plutei. The non-fertilizable condition is not due to the death of the egg but to a changed physiological condition induced by the initiatory action of the butyric acid treatment. Whatever the changes brought about, all of the conditions mentioned above are accompanied by the loss of substances from the egg, if indeed the condition is not a result of the loss of some substance or substances that are necessary for fertilization. This loss may be due either to the escape from the egg of the necessary substance or to new chemical combinations within the egg made possible by the disturbance of its physiological equilibrium.

It is highly interesting to note that a qualitative and possibly a quantitative test for one substance at least has been discovered by F. R. Lillie. This substance fertilizin is continually liberated from the egg into the surrounding sea-water as long as it remains in a fertilizable condition and as pointed out on page 276 it has been found to afford an absolute test (using a fresh sperm suspension as an indicator) of the capacity of sea-urchin eggs for fertilization in seven analyzed cases and will probably be found to hold for all cases in *Arbacia*, as it is evidently an indicator of a generalized condition.

An analogous condition to that in *Arbacia*, of the loss of substances from the egg and the consequent lack of capacity for fertilization, has been found in other forms than the sea-urchin, though in no other forms than *Arbacia* and *Nereis* has a qualitative test for a substance been found. E. E. Just ('15) has discovered that if eggs from *Platynereis* are collected in sea-water they cannot be fertilized. This is due to some effect of the sea-water in causing the loss of necessary substances for fertilization either through diffusion from the egg or through new chemical combinations within the egg. If only a few drops of sea-water come in contact with the egg they will not fertilize despite the fact that sperm penetrate the eggs and produce weak changes characteristic of fertilization: the proper egg substances for the intiatory reactions are not available.

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Miss Allyn ('12) found that any substance that had a tendency to initiate development in *Chætopterus* was detrimental to fertilization. She noted that the tendency was not for the exclusion of spermatozoa but rather for the production of polyspermy. The aberrant type of cleavage became more accentuated as the exposure to the initiatory agent was prolonged and would probably have entirely prevented fertilization with a slightly longer exposure. In this case the initiatory agents induced only a partial completeness of the reactions and as time went on fertilization became less and less possible, indicating the gradual loss of some substance whose presence is indispensable for fertilization. *Arbacia* eggs behave in essentially the same manner if over-exposed to butyric acid; polyspermy appears if insemination is carried out shortly after the exposure, but if the eggs remain standing in sea-water for a few hours insemination has no effect.

Just how hypertonic sea-water is effective we are not able to say, but the writer is inclined to the views of R. S. Lillie that a properly timed exposure to any substance that lowers permeability (unless it be decidedly toxic) allows of the combination of the substances within the egg that starts off the developmental reactions. If these changes are nearly enough the normal, development is quite complete up to certain stages in the life of the organism. The question, however, to which these experiments relate is the capacity for fertilization after activation by hypertonic sea-water.

The quantitative reaction idea has been pointed out quite freely in this and a preceding paper in considering the initiation of development, and certain instances of all phases of this process are to be had not only from the experiments themselves but also from the preserved material. From the purely parthenogenetic standpoint the ideas of the qualitative specificity of hypertonic sea-water as a factor in the initiation of development have been dealt a death blow by the experiments of R. S. Lillie ('15) on starfish eggs, and the purely quantitative idea substituted for the explanation. Lillie finds that not only can starfish development be initiated by a preliminary treatment with butyric acid followed by hypertonic sea-water, but also that a secondary

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treatment with butyric acid or warm sea-water or cyanide sea-water may be substituted for the hypertonic sea-water, or that both the preliminary and secondary treatments with warm sea-water may accomplish the same results. From inference any agent or combination of agents that cause a quantitative fulfillment of the reactions lead to the same end.

When a spermatozoön fertilizes an egg the optimum stimulation has been given to the egg, and we assume that the initiatory reactions have been quantitatively complete. But certainly in the initiation by artificial agents some of the eggs will not have been given the optimum conditions and will not have completed their reactions. If we can think in terms of chemical substances we will suppose that part of these have been left uncombined that are yet capable of some reaction with a spermatozoön if it but gain entrance to the egg. Consequently just so far as these fundamental reactions have been incomplete the spermatozoön is capable of asserting its latent stimulus for the activation processes. That fertilization is not complete in such cases is shown by the very irregular type of cleavage and the small per cent. of swimming larvæ and the poor quality of these, just as in a parthenogenetic treatment the same results are encountered if an exposure above or below the optimum one is given. If however the initiatory agent employed, has been affective enough to cause the production of good membranes (butyric acid), or has resulted in segmentation of the egg, the initiatory, reactive, egg substances appear to have been pretty well used up and so far as these experiments show there seems to be no tendency for a regeneration on the part of an egg of the fertilizable condition. The blastomeres of an egg appear to have no capacity, or at the most very little, to react with a spermatozoön. It has been pointed out above that out of hundreds of these isolated blastomeres never has one been able to produce a swimming larva, as a result of fertilization; and sections have revealed but one absolute case of sperm aster formation after penetration. One activation seems to preclude all others.

Fertilization appears to be due to an agent, spermatozoön, that initiates development by producing a condition that permits of the interaction of certain substances within the egg,

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which union curtails any further possibility of a subsequent fertilization. If the same conditions are produced by artificial parthenogenesis the same lack of the capacity for fertilization is evident.

Whether the lack of this capacity is due to the absence of one substance or of several substances, and whether the substance called fertilizin is the essential one or is only an indication of a certain physiological state of the egg is unknown. But in every case, so far examined, fertilizin has been present if the eggs could be fertilized and has been absent if there was no capacity for fertilization.

These results are weighty argument, if not definite proofs, against any idea of a sperm-borne substance that is necessary for the initiation of and the continuation of development. There is no evidence that the spermatozoön imparts a "lysin-like" substance that produces cytolysis within the egg and later carries into the egg a "corrective agent" to check the imposed cytolysis. The egg possesses all the substances it needs for development.

VI. Summary.

1. If *Arbacia* eggs are exposed to a weaker and a stronger concentration of sea-water for varying lengths of time a perceptible gradient of effectiveness from the entire absence of any reaction to an essentially normal parthenogenetic reaction is apparent.

2. The effective exposure is accompanied by a perceptible condition of increased permeability and substances escape from the eggs.

3. The superposition of insemination on the optimum hypertonic treatment does not increase the percentage of development.

4. Eggs when exposed to hypertonic sea-water for two hours usually cannot be fertilized; and in such a condition fertilizin cannot be detected by any known method. If however there yet remains a certain capacity for fertilization, fertilizin can be detected by its sperm agglutinating properties.

5. If *Arbacia* eggs are exposed to activating agents the permeability of the egg is increased, substances are lost from it and fertilization is impossible.

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6. If eggs are sectioned, after insemination subsequent to an exposure to the weaker hypertonic solution, a gradient of activity of spermatozoa parallels, to a certain extent, the length of the exposure to hypertonic sea-water.

7. If eggs are sectioned after insemination, subsequent to an exposure to the stronger hypertonic solution, large numbers of eggs are seen to have several spermatozoa within their boundaries; but they usually do not exhibit any signs of activity; many are undergoing degeneration.

8. Sperm, in gaining extrance to an egg, are pulled out into a long thin chromatin band situated in the protoplasmic cone produced by the protoplasm of the egg.

9. If a pure culture of the first two cells, produced by cleavage, are inseminated, sperm enter, remain entirely quiescent, and may even undergo disintegration. They do not fertilize the blastomeres.

10. These experiments furnish no evidence for, but are contrary to, any view that a spermatozoön carries a substance into the egg that is necessary for development. There is no evidence of a sperm-borne "lysin-like" substance nor a secondary "corrective agent." The egg possesses all the essentials for development.

HULL ZOÖLOGICAL LABORATORIES,
March 28, 1917.

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THE EFFECT OF STARVATION ON THE WING DEVELOPMENT OF MICROSIPHUM DESTRUCTOR.

LOUISE H. GREGORY.

As is the case with aphids in general, the life history of *Microsiphum destructor*, the green pea-aphid, shows a definite relation to the environment. Dimorphism is common. Winged and wingless individuals appear in varying numbers at different stages in the life history, the wingless forms predominating in the early part of the reproductive history, the winged forms increasing in number toward the end of the summer. While it is generally accepted that external conditions probably control the appearance of wings, very little data has been offered as proof. For a number of years, at the suggestion of Professor Morgan, I have experimented with the effect of starvation on the production of winged forms in the families of the green pea-aphid. I am glad to express my appreciation of Professor Morgan's helpful criticisms.

In starting all experiments, a single individual, selected to produce the stock family, was isolated on a young pea plant in a small pot and covered with a lamp chimney, the top of which was covered with gauze to prevent the escape of the aphid and the confusion of families. This method of isolation was used throughout the investigation and proved most successful, the plants growing normally, sufficient air entering at the top of the chimney. From the family of the stock aphid, some young were isolated and used as control lines, others were removed daily from the plant and starved for periods of different lengths, twenty-four hours, twelve hours and eight hours. Experience proved that fewer deaths resulted if the aphids were starved eight hours daily. After the period of fasting the starved aphids were isolated and their offspring observed. As will be clear later, the effect of starvation on wing development is not apparent in the individuals starved, but in their offspring.

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TABLE I.

Starvation Exp. I. — Parent non-winged. Both series from one parent. A2 series younger. — *Control.*

Date 1913–14, winter. — 1913–14.

No. Exp.	Hours Starved.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
A1 series	30					Starved—						*Winter*
No. 2		14	49	63		4 days—av. 7½ h.	No. 2	31	14	45		
No. 3		6	34	40		per day—	No. 4	43	4	47		
No. 6		14	73	87			No. 6	28	23	51		
No. 7		25	61	86			No. 8	32	9	41		
No. 8		39	37	76			No. 10	41	3	44		
		—	—	—		Av. family		—	—	—		Av. family
		98	254	352	71+	70+		175	53	228	23%	45.4
A2 series	32¾					4 days starved						
No. 2		30	0	30		av. 8.10 daily						
No. 3		27	23	50								
No. 4		34	40	74								
No. 5		23	54	77								
No. 6		24	43	67								
No. 7		35	43	78								
No. 8		16	20	36								
No. 9		25	26	51								
		—	—	—								
		214	249	463	53.8	Av. family 57						
Grand Total		312	503	815	61.1	Av. family 62						

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The first experiments were carried on during the winter of 1913–1914 in the greenhouse at Columbia University (Table I.). Five young of a wingless mother were deprived of food 7½ hours daily for four days. These (A1 series) produced a total of 352 young, 254 or 71 per cent. of which were winged. In the second series (A2) younger members of the same family, 8 aphids were starved 8 hours daily for four days. The total number of offspring in the eight families was 463, of which 249 or 53 per cent. were winged. In the control series of a total of 228 (young of five families) 23 per cent. were winged showing a difference of 48 per cent. if compared with the A1 series and of 20 per cent. with the A2 series. If the entire A series is considered as a whole, among the young of 13 families 61 per cent. were winged. Apparently the lack of food has had its effect on the second generation, none of the starved individuals developing wings.

The experiments were repeated at Woods Hole in the summer of 1915 (Table II.). Again the young of a wingless mother were used for the experiment and control. The families of seven wingless females starved 45 hours, were observed and out of a total of 188 young, one winged form only appeared. The seven families of the control series showed 3 winged individuals. Apparently the effect of fasting was overcome in some way.

Considering that the atmospheric conditions had been unusually favorable for the development of the peas, the weather having been very hot with much humidity, I concluded the food had been sufficiently rich to overcome any adverse effects of starvation, and that in order to induce the effects of starvation, it would be necessary to increase the number of starvation periods. Consequently the experiments were repeated the following summer in Princeton, Mass. (Table III.).

Five young starved 60 hours produced a total of 254 offspring, 30 per cent. of which were winged. Ten individuals forming the control series produced 514 young, of which 56, or 9.7 per cent., were winged. Here again starvation has had its effect.

Summarizing all the experiments on wingless parents, it is seen in Table III., that 25 starved individuals produced 1,257 young, 46 per cent. of which had wings: that 22 normal indi-

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TABLE II.

1915.		*Starvation Exp. II.* Parent non-winged.					1915.		*Control.* Parent non-winged.			
No. Exp.	Hours Starved.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-Winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
A_2	45					Woods Hole	A_1					Woods Hole
(1)		37	0	37		Plants luxuriant	(1)	38	0	38		
(3)		15	0	15			(2)	71	3	74		
(4)		18	0	18			(3)	49	0	49		
(5)		32	0	32			(4)	39	0	39		
(6)		36	0	36			(5)	48	0	48		
(7)		18	0	18			(6)	34	0	34		
(10)		31	1	32			(10)	65	0	65		
		187	1	188		Av. family 26.8		344	3	282	1.	Av. family 39.5

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viduals produced 1,149 young, 9.7 per cent. of which were winged. The starved mothers had about three times as many winged young as the control. Since all the families were subjected to the same conditions of temperature and humidity, the variation in the results must be due to the only factor in which they differ, namely the food supply.

Temperature undoubtedly may effect the production of wings as Ewing[1] has shown in his experiments with *Aphis avenæ*. He found that by keeping the temperature constant at 65° F., he could prevent the appearance of any winged forms. At lower and higher temperatures winged forms appeared. These results might be interpreted as due *not* to the direct effect of temperature on the development of wings but on the growth of the plants or the food supply and thus indirectly affecting the metabolism of the individuals.

If the summer experiments are compared with those in the winter, it will be observed that the percentage of winged forms is less in both starved and control lines. However the percentage of increase in number of winged forms in the starved lines remains constant. In the summer as in the winter there are three times as many winged young in the starved lines as in the control. I see no reason why the temperature might not have been effective indirectly in the lessening of the wing production in both starved and control summer lines. The atmosphere of the greenhouse was practically constant, while the summer experiments were carried on out of doors and were subject to all the daily changes in temperature and moisture. The actual difference in the development of wings at any one time must have been due to the difference in food supply, temperature having no part. I believe that if aphids were starved for long periods and kept at a constant temperature of 65° F., Ewing's optimum temperature, winged individuals would appear.

Overcrowding on the plants has been suggested as a possible cause of wing development. In nature this might be true, the results however being due to a lessening of the food supply. In my experiments there was no overcrowding as the young were

[1] Ewing, H. E., "87 Generations in Parthenogenetic Pure Line of *Aphis avenæ*," BIOL. BULL., Vol. XXXI., No. 2, 1916.

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TABLE III.

Starvation Exp. III. 1916 July. Parent non-winged.							*Control.* 1916 July. Parent non-winged.					
No. Exp.	Hours Starved.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
	60 hrs.					Princeton, Mass.						
1		35	51	86		Alt. 1100 ft.	2	47	0	47		
3		23	11	34		Atmosphere dry	4	56	3	59		
5		50	1	51		and cool	6	33	5	38		
9		39	14	53		Experiments in	8	63	0	63		
11		29	1	30		open	10	61	0	61		
							12	58	0	58		
		176	78	254	30.7	Av. family 50+	14	79	11	90		
							16	37	35	72		
							18	42	0	42		
							20	42	2	44		
								518	56	574	9.7	Av. family 57.
						Summary						
1913–14 13 fam.		312	503	815	61.7	*Winter* Starved 30–32 hours. Av. family 62.	1913–14 5 fam.	175	53	228	23	*Winter* Av. family 45.
1915 7 fam.		187	1	188		*Summer* Woods Hole Starved 45 hours.	1915 7 fam.	344	3	347		*Summer*
1916 5 fam.		176	78	254	30.7	*Summer* Princeton Starved 60 hours.	1916 10 fam.	518	56	574	9.7	*Summer*
Total 25 fam.		675	582	1257	46.3	Av. fam. 50.2	Total 22 fam.	1037	112	1149	9.7	Av. fam. 52.2

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TABLE IV.

1913–14. *Starved.* Parent winged.						1913–14. *Control.* Parent winged.					
No. Exp.	No. Non-Winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.	No. Exp.	No. Non-winged Offspring.	No. Winged Offspring.	Total Family.	Per Cent. Winged.	Remarks.
Winged A[1] 4....	65	2	67	.3	Starved 30 h.	1.....	15	0	15		
	—	–	—			3.....	26	0	26		
	65	2	67			5.....	25	4	29		
						7.....	28	0	28		
						9.....	27	0	27		
							—	–	—		
							121	4	125	3.	Av. family 25.
1914–15. Winged G (5).	29	0	29	0	♀ died young.	No. controls					
(6).	15	0	15		Starved 45 h.						
	—		—								
	44		44								
Non-winged— G (2).....	11	8									
(8).....	44	16			" "	"					
(9).....	14	15									
	—	—									
	69	29	98	29.							

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removed from the plants as soon as it was possible to determine the presence or absence of wing buds.

It has been suggested that young produced in the early part of the reproductive life of the mother would be better nourished and fewer wings would develop than among those born at the end of the period of reproduction. A consideration of the A1 and A2 series of Table I. seems to disprove this, as more wings developed in the families of the older members.

It might also be suggested that the effect of starvation was to reduce the size of the families and consequently the percentage of winged forms might be raised. In some cases the size of families of the starved mothers was smaller than that of the control, in other cases it was greater. The average sized family was 50.2 in the starved lines and 52.2 in the controls. The difference is not large enough to be important.

A few experiments were carried on with starved winged mothers. These are difficult to obtain, since the number normally produced is low and since it is impossible to distinguish wing buds at the early period when starvation is begun, thus the chances for finding winged forms among the starved young are slight. The control experiments (Table IV.), show that of the young of five winged mothers only 3 per cent. were winged. In the few families of starved winged mothers the percentage is less, often none of the young develop wings In passing it might be noted that in the G series (Table IV.) no. 5 and no. 6 winged individuals starved 45 hours produced no winged young, while their wingless sisters nos. 2, 8, 9, produced 29 per cent. winged forms. Apparently starvation has little effect on winged mothers. Teleologically this result would be expected. There would be no need for more winged forms in nature, since the parent had migrated to a fresh food supply.

In conclusion it seems most probable that the lessening of the food supply is the primary factor in determining the development of wings in the offspring of wingless mothers. The wing anlage appears to be present in all the parthenogenetic females and depends directly upon the condition of the food supply of the mother for its stimulation or suppression of development.

COLUMBIA UNIVERSITY,
June, 1916.

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Vol. XXXIII. *November, 1917.* *No. 5.*

BIOLOGICAL BULLETIN

THE PRODUCTION OF FUNCTIONAL AND RUDIMENTARY SPERMATOZOA IN ROTIFERS.[1]

D. D. WHITNEY.

Marked dimorphism in the spermatozoa of a few animals has been briefly recorded by several observers, but no detailed study has been made of such forms to determine how the two kinds of spermatozoa are formed nor has there been offered an explanation as to their probable functions. While looking at the plates in "The Rotifera," by Hudson and Goss, I noticed sketches of the two dimorphic spermatozoa of the marine rotifer, *Brachionus mulleri*. As this rotifer occurs in countless numbers in a salt-water lake near Lincoln, Neb., an excellent opportunity was offered to reëxamine it in an attempt to find an explanation for the production of the two kinds of spermatozoa. After reëxamining the spermatozoa of this rotifer and confirming the observations of Hudson and Goss the spermatozoa of eight other species of rotifers were examined and were found also to be composed of two kinds. Thus indicating that this phenomenon of dimorphism of spermatozoa may be general for all the species of the whole group of rotifers which produce male individuals.

The technique of examining the spermatozoa is very simple. Mature free swimming males were placed on a slide in a drop of the culture water and covered with a cover glass. Then by pressure the males were forced to eject the contents of the single testis into the water. The two kinds of spermatozoa could be readily seen even with the low powers of the microscope and were as easily counted. Early stages in the development of the spermatozoa as far back as the early spermatids were studied by crushing the eggs containing the unhatched immature males. All stages of development from the earliest spermatid up through

[1] Studies from the Zoölogical Laboratory, The University of Nebraska, No. 117.

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to a mature spermatozoön were easily seen, especially in the species of rotifers whose males form only a few spermatozoa.

Brachionus mulleri, the salt-water rotifer, was the first species examined and because of the relatively small number of spermatozoa produced by the males it proved to be the most favorable form in which to see the early stages in the development of the spermatozoa as well as for making accurate counts. The motile spermatozoa were extraordinarily large and measured about one third the total length of the male individual that produced them. Probably on account of the large size of the spermatozoa a male is physically unable to form very many. These motile spermatozoa were long and somewhat slender with a pointed anterior end. The posterior two-thirds of the spermatozoön is very motile and has a prominent, undulating, median, and dorsal membrane which aids greatly in the locomotion. The sketch of a mature spermatozoön in Fig. 1, *E*, is a dorsal view and does not show adequately this membrane. These spermatozoa swim freely about in the water for a short time after they are forced out from the body of the male.

The spindle-shaped spermatozoa are incapable of any locomotion. They are uniform in shape and never change their form. They are very much smaller than the motile spermatozoa. Fig. 2, *D*, shows several of these. As the tissues of the males are very transparent both kinds of these spermatozoa may be readily seen inside the testis. The spindle-shaped spermatozoa are usually found collected together near the posterior end of the testis, although sometimes only a few are thus collected together while the remainder are scattered throughout the testis among the motile spermatozoa.

When the immature males are crushed the spermatids still united in masses separate readily from the general debris. Various stages in the development of the spermatids and spermatozoa may be seen in accordance with the stage of development of the male. All spermatids or immature spermatozoa in each male are generally found in the same stage of development.

The relative number of the two kinds of spermatozoa varied widely when counts were made from the mature free swimming males taken from a general culture of rotifers. It is very prob-

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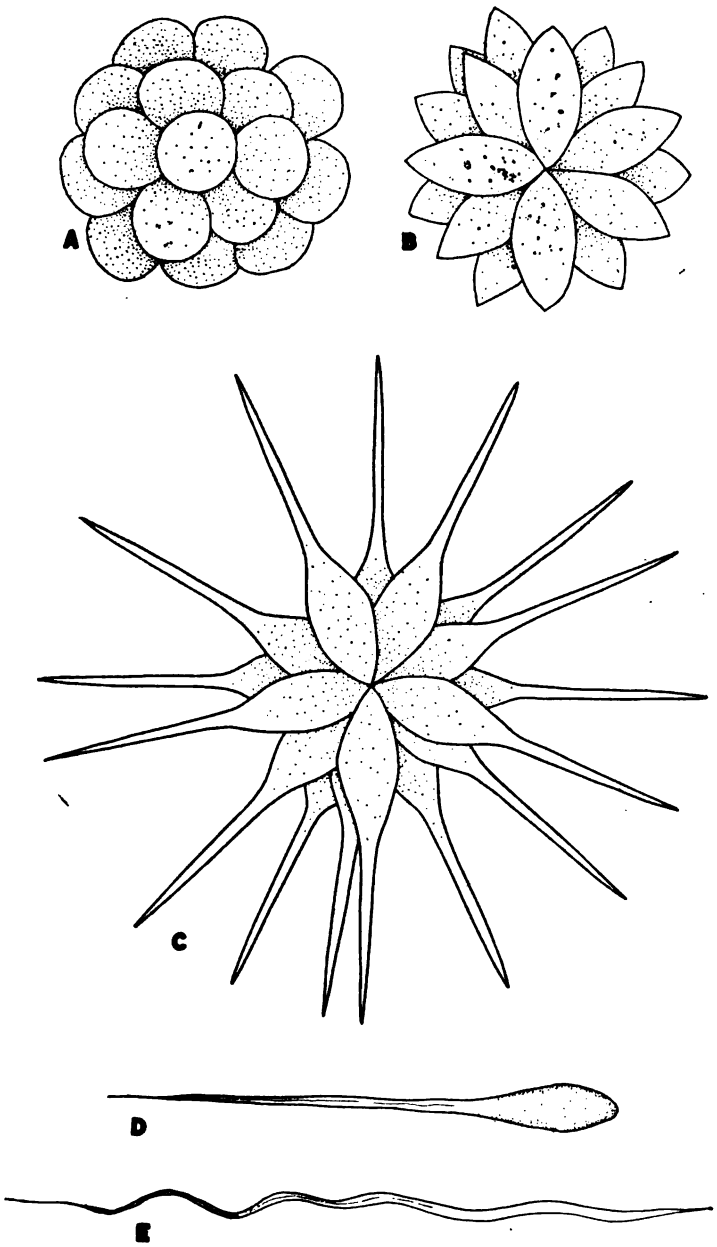

FIG. 1. Formation of the functional spermatozoa in *Brachionus mulleri*. *A*, young spermatids; *B*, older spermatids; *C*, young immature spermatozoa; *D*, older immature spermatozoön; *E*, mature, motile, and functional spermatozoön.

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able that many of these males had already paired with females and consequently had shed some of their spermatozoa. This would explain the variability of the spermatozoa counts in the first twenty-one males recorded in Table I.

TABLE I.

Brachionus mulleri. Showing the relative proportional number of the two kinds of spermatozoa found in each testis of thirty male individuals. The rudimentary spermatozoa were never found as numerous as the functional spermatozoa in any testis of a young male. In the immature males in which none of the spermatozoa had been fully formed or shed the larger spermatids that ultimately develop into the functional spermatozoa were found to be exactly twice as numerous as the smaller spermatids that ultimately develop into the rudimentary spermatozoa.

Serial Number.	State of Development of Males.	Number of Functional Spermatids or Spermatozoa in Testis.	Number of Rudimentary Spermatids or Spermatozoa in Testis.	Serial Number.	State of Development of Males.	Number of Functional Spermatids or Spermatozoa in Testis.	Number of Rudimentary Spermatids or Spermatozoa in Testis.
1	Mature	50	26	16	Mature	5	0
2	"	48	—	17	"	24	8
3	"	—	12	18	"	9	10
4	"	—	0	19	"	28	10
5	"	6	2	20	"	18	6
6	"	—	15	21	"	60	25
7	"	2	1	22	Immature	38	18
8	"	20	11	23	"	70–80	38–40
9	"	—	18	24	"	40	18–20
10	"	22	13	25	"	40	18–20
11	"	4	0	26	"	60	32
12	"	15	5	27	"	64	32
13	"	56	35	28	"	40	20
14	"	28	11	29	"	56	28
15	"	40	22	30	"	48	24

When immature males were crushed one could readily count all the spermatids of both kinds and could definitely determine the relative number of each. It was seen at once when all spermatids were counted that the two kinds of spermatids that develop into the two kinds of spermatozoa did not occur in equal numbers. The large spermatids which ultimately develop into the large motile spermatozoa were found to be exactly twice as numerous as the smaller spermatids which ultimately develop into the small spindle-shaped spermatozoa. This is clearly shown in the last few males recorded in Table I. Figs. 1 and 2

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show a series of the various stages of development from early spermatids to mature spermatozoa of both kinds of these dimorphic spermatozoa.

Mature males of *Hydatina senta* were crushed and examined and were found also to possess the two kinds of spermatozoa as markedly dimorphic as those in *Brachionus mulleri*. Many more spermatozoa are formed in the testis and consequently the counts could not be as readily or as accurately made as in *Brachionus mulleri*. Counts enough, however, were made to show that the large motile spermatozoa were about twice as numerous as the smaller immotile spindle-shaped spermatozoa.

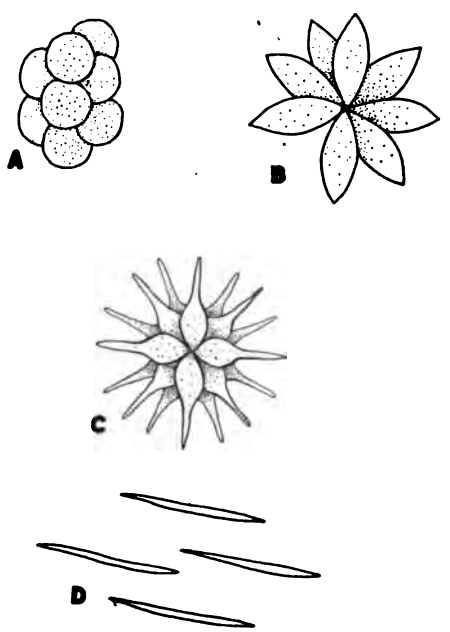

FIG. 2. Formation of the rudimentary spermatozoa in *Brachionus mulleri*. *A*, young spermatids; *B*, older spermatids; *C*, young immature spermatozoa; *D*, mature, immotile, and rudimentary spermatozoa.

The counts are shown in Table II. and drawings of the two kinds of spermatozoa are shown in Fig. 5.

The spermatozoa were examined next of the large viviparous rotifer, *Asplanchna amphora*, and although no very accurate counts could be made the two kinds of spermatozoa were con-

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spicuously present. The highest count for the motile spermatozoa was about 200, while the highest count of the immotile spindle-shaped spermatozoa was about 100. These counts were made from males which were isolated at birth before they

TABLE II.

Hydatina senta. Showing the relative proportional number of the mature dimorphic spermatozoa found in each testis. In general the large motile spermatozoa were twice as numerous as the small spindle-shaped spermatozoa.

Serial No.	State of Development of Males.	Number of Functional Spermatozoa in Testis.	Number of Rudimentary Spermatozoa in Testis.	Serial No.	State of Development of Males.	Number of Functional Spermatozoa in Testis.	Number of Rudimentary Spermatozoa in Testis.
1	Mature	60	18	8	Mature	—	50–60
2	"	—	24	9	"	50–60	25–30
3	"	72	26	10	"	0	5
4	"	160–200	—	11	"	20	18
5	"	130	44	12	"	186	—
6	"	—	40	13	"	90	52
7	"	—	30				

had had an opportunity to pair with females and to shed their spermatozoa. They show that the motile spermatozoa are about twice as numerous as the immotile kind. Drawings and counts of these two kinds of spermatozoa are shown in Fig. 3 and Table III.

TABLE III.

Asplanchna amphora. Showing the relative proportional number of the mature dimorphic spermatozoa found in each testis. In general the large motile spermatozoa were twice as numerous as the small spindle-shaped spermatozoa.

Serial Number	State of Development of Males.	Number of Functional Spermatozoa in Testis.	Number of Rudimentary Spermatozoa in Testis.
1	Mature	200	50
2	"	200	60
3	"	—	104
4	"	160	75

In addition to the spermatozoa of these three species of rotifers already described the spermatozoa of six other species of rotifers were carefully examined. These species were *Polyarthra platyptera*, *Diglena catellina*, *Euchlanis dilatata*, *Metopidia lepadella*, *Brachionus urceolaris* and *Brachionus bakeri*. In all cases the

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two kinds of spermatozoa were found and the larger motile ones were more numerous than the smaller immotile ones. Drawings of these spermatozoa are shown in Figs. 4, 6, 7, 8, 9 and 10.

Morgan has found in the study of the spermatogenesis of some of the phylloxerans that the secondary spermatocytes are of two kinds—the normal and the rudimentary. The normal spermatocytes divide again to form the spermatids, each of which develops later into a functional spermatozoön. The rudimentary spermatocytes do not divide again and do not form spermatids or spermatozoa. They merely persist for a time as rudimentary secondary spermatocytes and then finally disintegrate and disappear. Morgan has also found the same phenomenon in some of the aphids, Stevens and von Baehr have also found it in the aphids, Meves has found it in *Vespa germanica*, Mark and Copeland have found it in *Vespa maculata*, and Lams has found it in an ant, *Campodinotus herculaneus*.

In the grand total count of all the spermatocytes and spermatids at the end of the final spermatocyte divisions in all of these forms enumerated in the preceding paragraph the normal spermatids should have been twice as numerous as the rudimentary spermatocytes. No counts, however, were made and consequently this point was not settled by this method but it was demonstrated in another manner by showing that only one spermatocyte division occurred in the rudimentary spermatocytes and two divisions occurred in the normal spermatocytes in the process of formation of the normal spermatozoa. In 1909 Morgan said in regard to the rotifer, *Hydatina senta*, which at the time had not been investigated. "Theoretically there should not be two spermatocyte divisions but only one true division. It remains to be seen whether this prediction proves true."

The rotifers have not only fulfilled but have exceeded Morgan's expectations! They not only produce normal spermatids and rudimentary secondary spermatocytes in the ratio of 2:1 as probably do these forms already mentioned, but they develop both of these into spermatozoa which are later shed by the males. Those of one kind are large, motile and probably functional while those of

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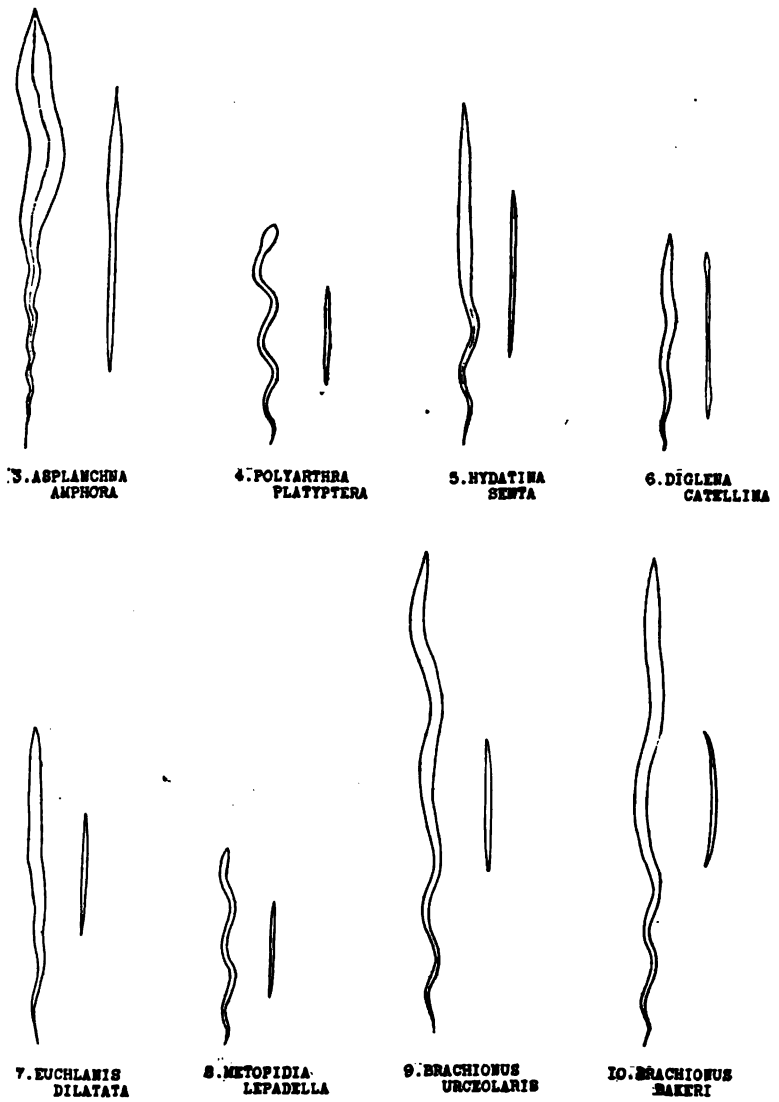

FIGS. 3–10. Dimorphic spermatozoa of eight species of rotifers showing the larger, motile, and functional spermatozoa and the smaller, immotile, and rudimentary spermatozoa. In all of these species the drawings were made of spermatozoa that were taken from mature free-swimming males except in Fig. 4 which were made of spermatozoa of unhatched immature males. All were drawn to same scale as Figs. 1–2.

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the other kind are small, immotile and probably rudimentary, and without function. The rudimentary spermatozoa must necessarily develop directly from the secondary spermatocytes without the occurrence of the second spermatocyte division in order to form the ratio of two normal spermatozoa to one rudimentary spermatozoön. If they developed from rudimentary spermatids which were formed by the division of the secondary spermatocytes the ratio would be one functional spermatozoön to one rudimentary spermatozoön. This interpretation of the observed ratios would therefore place the rotifers in the same category as the other animals already mentioned as far as the formation of normal spermatids and of rudimentary secondary spermatocytes are concerned. They would, however, have this point of difference, viz.: that the degree of degeneration of these rudimentary secondary spermatocytes has not progressed as far as to render such spermatocytes incapable of developing directly into degenerate spermatids and spermatozoa of a certain kind, as is the case of the other forms studied.

In the mature parthenogenetic egg that develops into the male individual there is the reduced number of chromosomes. This contains one half as many chromosomes as are found in the parthenogenetic egg that develops into the female. This fact was made out in *Hydatina senta* but the exact number of chromosomes occurring in each kind of egg has never been determined. If the male in the beginning has already the reduced number of chromosomes no further reduction of chromosomes is necessary in the formation of the spermatozoa. The race, however, may have retained the former ancestral process of performing the first spermatocyte division, regardless of the results, in the formation of the secondary spermatocytes but as there is no general reduction of chromosomes to be made at this stage presumably none occurs. One half of the secondary spermatocytes very likely contain fewer or perhaps no chromosomes at all and consequently are very degenerate and rudimentary and without a second division develop directly into degenerate spermatids and spermatozoa.

The male parthenogenetic eggs are the ones that are fertilized as has been determined by Maupas, Lauterborn, Whitney, and

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Shull and after fertilization always develop into females but

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THE SPERMATOCYTIC DIVISIONS OF LEPTOCORIS HÆMATOLOMA.[1]

LELIA T. PORTER.

INTRODUCTION.

The spermatogenesis of *Leptocoris hæmatoloma* (family Coreidæ) very closely resembles that of *Anasa tristis*, as described by Paulmier. During the past few years it has been used extensively here in the course on cytology to demonstrate the behavior of the X-chromosome. As a matter of record, it seems worth while to give a very brief account of the spermatocytic divisions. Furthermore, the material is very fine for the study of tetrad formation, and it is hoped later to publish an account of this process.

The material on which these observations were made was collected in November and December, 1914, on the plant *Cardiospermum halicacabum*, the balloon vine which is very common in this vicinity. It may be of interest, however, to know that this is the only plant on which we have found this bug. The testes, which are large yellow pear-shaped bodies located at the base of the third pair of walking legs were dissected out in Ringer's solution and fixed immediately in Bouin's fluid. They were stained with iron hæmatoxylin and counterstained with eosin and light-green. Other fixing agents were used, but this proved to be the best.

The material is excellent for the study of spermatogenesis, as the chromosomes are not numerous, and vary greatly in size. It is possible to follow accurately the courses of the smallest, largest and accessory chromosomes throughout their entire history.

The spermatogonial complex of *Leptocoris hæmatoloma* consists of thirteen chromosomes (Figs. 1 and 2). These vary in size, but can be classified in three groups. The members of two pairs

[1] Contributions from the Zoölogical Laboratory of the University of Texas, No. 138.

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are quite large, three pairs are of medium size, while the sixth pair and the accessory chromosome are rather small. On account of their size and shape, one has little difficulty in recognizing the members of the various pairs.

After a series of successive spermatogonial divisions, the thirteen chromosomes pass through the characteristic synaptic and growth stages, giving rise to six tetrads and one dyad, typical of the primary spermatocyte. These tetrads vary greatly in size, the largest and smallest being easily recognized in Figs. 3 and 4. Three of the tetrads approximate each other very closely, and the remaining one is slightly smaller than the largest. The dyad representing the accessory chromosome lies close to the nuclear wall in Figs. 3 and 4, but I have no reason to believe that this is necessarily characteristic. Figs. 3 and 4 were selected because in each the entire number is seen, though scattered irregularly throughout the nucleus. At this stage the nuclear membrane is intact and the cell itself has increased enormously in size.

The tetrads are then drawn into the central plate and there is no question as to their final arrangement Fig. 5 shows the typical position of the various chromosomes in the central plate of the first spermatocyte. I have examined numbers of cells and at this stage have always found the six chromosomes arranged in a ring with the smallest in the center. The accessory chromosome always lies in the circle. Paulmier has found this to be the case in *Podisus*, *Lygæus* and *Chariesterus*, but Henking finds no such peculiar arrangement in *Pyrrochoris*.

First Spermatocytic Division.

The spindle of the primary spermatocyte is ordinarily rather broad but as the chromosomes begin to pull apart, it becomes in many cases, long and slender. The fibers of the spindle converge at the poles, but no centrosomes could be found in any of these stages. This may have been due to the stains used. The chromosomes in the metaphase plate do not divide synchronously. The accessory tends to divide precociously (Figs. 8 and 9) and is generally followed by the smallest chromosome (Figs. 6 and 7). As the accessory advances toward the poles,

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the larger chromosomes are seen to be pulled apart. As they divide and separate they are usually still connected by two thick threads (Figs. 6, 7, 9). These fibers stain deeply with hæmatoxylin but are lost sight of as the chromosomes are pulled farther apart and the cell wall begins to constrict. There is no sign of a zwischenkörper as described by Paulmier for Anasa. Each of the two resulting secondary spermatocytes (Figs. 11, 12, 13) contains six chromosomes in the form of dyads and one monad, the accessory. There is no trouble identifying the accessory because of its size and position.

Second Spermatocytic Division.

The second division follows directly upon the first without any resting stage. In the equatorial plate of the second division the chromosomes are arranged in the characteristic ring of six with the smallest chromosome in the center. In the case of *Anasa tristis* Paulmier finds no such regular arrangement though he says the small chromosome generally lies in the center. The chromosomes now arrange themselves on the middle of the spindle and very soon the accessory is seen to leave the group and pass undivided in advance of the others toward one of the poles (Figs. 14, 16, 17, 18, 19, 20). In *Anasa* the accessory lags, and is not drawn toward one of the poles until the other chromosomes have practically come to the end of their movement. The remaining dyads then divide apparently in the same order as for the first division, the smallest leading (Figs. 15, 17, 19). These then pass to opposite poles and the cell constricts in the middle. The resulting cells are smaller than those of the first division, half of them containing seven chromosomes, and half six (Figs. 21, 22, 23, 14). The chromosomes of these spermatids have the same characteristic arrangement as those of the primary and secondary spermatocytes, one half of them having a ring of six with a small one in the center and the other a ring of five with the small one also in center. There is a typical massing of the chromosomes in the spermatid before the nuclear wall is formed.

The spindle of the second spermatocyte is similar in shape to that of the first, being rather broad and thick at the initiation

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of division, and as the chromosomes are pulled apart, taking on a slender elongated appearance.

In conclusion it may be suggested that the female has two X-chromosomes, or fourteen in all. Wilson reports that *Leptocoris trivittatus* has fourteen in the female and thirteen in the male.

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Explanation of Plate I.

All drawings were made with the aid of a camera lucida at table level. A Zeiss microscope with compensating 12 ocular and 2 mm. oil immersion objective was used. The drawings have been reduced one third off.

1, 2. Equatorial plate of spermatogonia.
3, 4. Tetrad stages, showing all chromosomes.
5. Polar view of first spermatocyte.
6–8. Side view of same.
9. Early anaphase of first division.
10. Side view of first spermatocyte division.
11–13. Polar view of second spermatocyte.
14–20. Second spermatocytic division.
21, 22. Spermatids, corresponding daughter cells.
23, 24. Spermatids, corresponding daughter cells.

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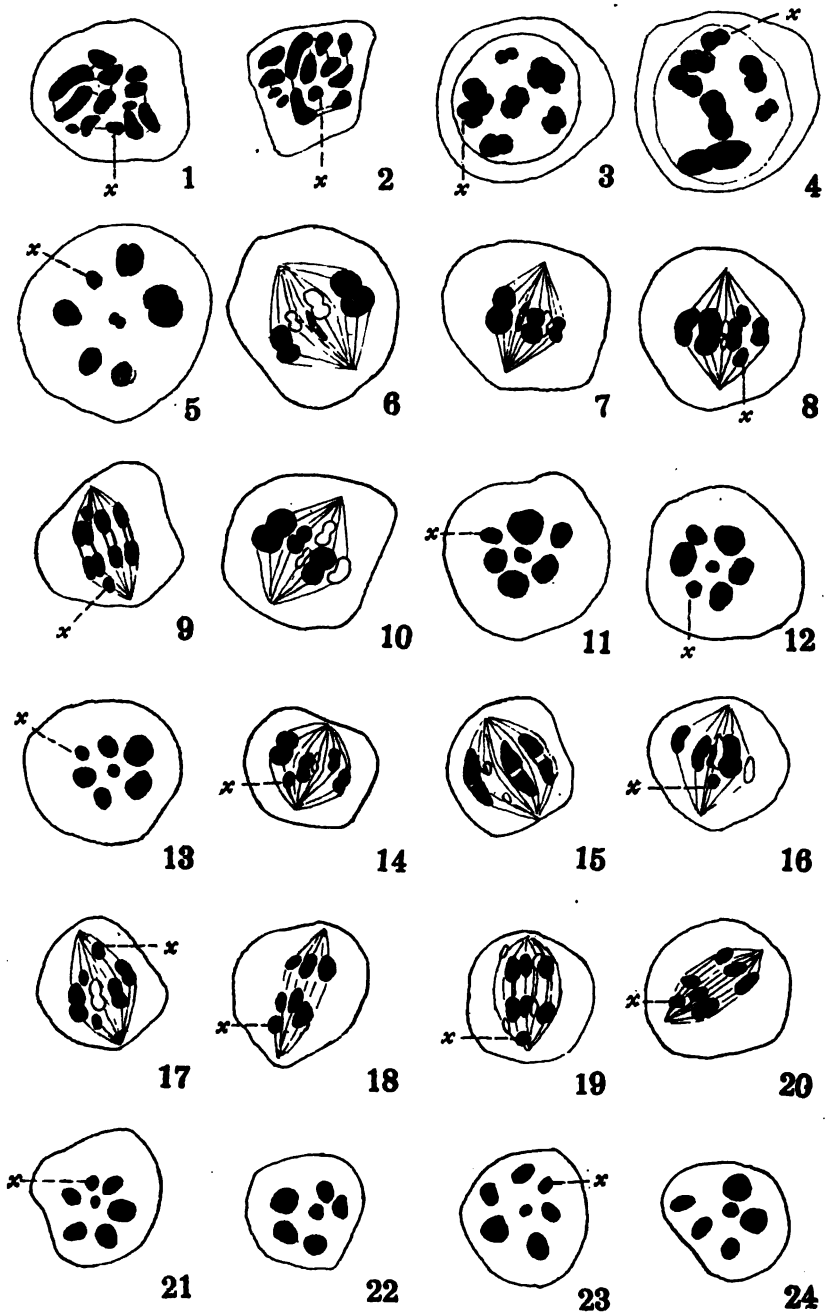

LELIA T. PORTER.

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THE FATE OF THE UNFERTILIZED EGG IN THE WHITE MOUSE.

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OSBORN ZOÖLOGICAL LABORATORY, YALE UNIVERSITY.

It has long been known that in mammals many more eggs are ovulated than develop into embryos, due in part to the failure of the spermatozoa to reach them, and perhaps more often to the inability of the eggs to implant themselves in the uterus. Such eggs are said to degenerate, but the story of their fate has been recorded in only a few instances. Since ovulation in the mouse is independent of copulation, this animal was selected for the investigation. It is natural to suppose that the process would be similar to the degeneration of the ovarian egg which has been prevented from leaving the ovary, the process termed follicular atresia.

The atresia of ovarian eggs in mammals has been described in detail by many investigators, but the degeneration of the unfertilized egg in the Fallopian tube and uterus has been given but little special study. Heape ('05) working on the domestic rabbit, mentions the finding of eggs in the tube which for some reason or other had not been fertilized and were in a process of degeneration. Hartman ('16), in his study of the early development of the American opossum, also refers to the great number of unfertilized eggs, approximately 50 per cent., which he found in the uterus, in an earlier or later stage of degeneration. He ventures the opinion that these eggs would eventually leave the uterus.

Students of atresia have usually recognized that there is an early disturbance of the nucleo-cytoplasmic relationship, although they have not always given it that name, and that it was followed by a rearrangement of the cytoplasm. Some writers however insist that a true parthenogenetic cleavage may take place, and continue in a more or less normal manner up to, in some cases,

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the ten or twelve cell stage before the degenerative processes get the upper hand.

The purpose of the present investigation is to describe step by step the degeneration, whether it be fragmentation or parthenogenetic development, of the unfertilized egg in the white mouse, and to compare the process with follicular atresia in the mouse and other mammals.

The work was undertaken at the suggestion of Prof. W. R. Coe, and done under his supervision. I am pleased to acknowledge his many valuable suggestions during the course of the investigation.

LITERATURE.

Kingery ('14) describes the method of atresia in the follicles of young mice. In his opinion the process is one of degenerative fragmentation only, that the spindle fibers of the second maturation spindle break, forming aster-like radiations and that later the achromatic fibers entirely disappear. Then the loose chromosomes form vesicles, and occasionally these fuse together to form larger vesicles. This disturbs the nucleo-cytoplasmic relationship, and an attempt to counter balance is made by the cytoplasm breaking up and surrounding the nuclei, the larger amount of cytoplasm enclosing the larger nuclear vesicles. In some cases the cytoplasmic fragments have no nucleus whatever. These fragments with or without nuclei are later absorbed, probably by the action of phagocytic cells of follicular origin.

Newman ('13) found that in the armadillo the first maturation spindle was formed in the ovary and that the egg then waited for ovulation. Eggs not located near the periphery of the ovary were not ovulated. In such cases Newman found that only a small percentage cut off the first polar body and that only three per cent. in several hundred cases gave off the second polar body, while ninety per cent. of the eggs were struck by a process of follicular atresia, which is either a cytolysis or a parthenogenetic development. He considers only the latter contingency. In such eggs there is first a casting out of the deutoplasmic mass from the formative cytoplasm. This he considers to be an act of rejuvenation on the part of the dying cell. Released from the burden of the yolk the cell is better

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able to carry on the natural processes. The abstrictions of the yolk often look like multicellular structures, but Newman thinks this appearance is due to fixation. Some of these yolk fragments which he calls cytoids contain deutoplasmic granules.

Newman finds numerous tri- and multipolar spindles. These, he thinks, would result in nests of nuclei without division of the cytoplasm. Sometimes instead of a single resting nucleus formed from the maturation spindle, two nuclei might be formed instead, without the extrusion of the polar body, and suggests that from these double nuclei, the multipolar spindles might be formed. As the number of chromosomes is more than the haploid number such spindles cannot be maturation spindles. In those cells with a true cleavage spindle, Newman is inclined to the view that no polar bodies have been given off. The process is in his opinion a true parthenogenesis, but development does not proceed beyond the eight cell stage and even at that time advancing degenerative processes are to be seen.

Heape ('05) found that in the domestic rabbit copulation acted as a stimulus to certain internal rearrangements which ended in severing the ovum from its source of nourishment. At about nine hours after copulation the two polar bodies are formed and ovulation takes place about an hour later. He says: "Once freed from the ovary the mature ovum is incapable of assimilating nutriment unless it be fertilized; if from any cause fertilization is not effected, the ovum quickly dies, although it is bathed in the nutrient material supplied by the maternal tissues; ova thus degenerating are from time to time to be seen in the Fallopian tubes." It is therefore necessary for spermatozoa to be at the top of the Fallopian tube because, unlike the condition in the mouse, the ovum is dehisced from the ovary without any discus cells to provide it with food.

Bonnet ('00) reviews the different theories of degeneration and takes the view that the various spindles figured by the exponents of the parthenogenetic idea are to be considered more or less abnormal maturation spindles, and not cleavage spindles.

Van der Stricht ('01), in his study of follicular atresia in the bat, comes to the conclusion that the oöcyte of the second order forms a cleavage spindle and divides parthenogenetically. He

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has traced an apparent normal cleavage as far as the six-cell stage. He also described multipolar spindles and occasionally two spindles in the same egg. The first polar body is occasionally seen to divide.

Kirkham ('07) gives an excellent review of the literature on the early development of mammalian eggs previous to 1907. The common occurrence of spindles in the polar body led him to conclude that the polar body would divide mitotically. Abnormal eggs containing tripolar spindles and in one case an egg containing two spindles were observed. Kirkham agrees with Sobotta ('95) and Rubaschkin ('06) that an egg never develops after the formation of the first polar body and the second polar spindle unless fertilization takes place, the egg degenerating within the ovary or in the Fallopian tube.

Athias ('09) also takes the view that the process is entirely one of degenerative fragmentation.

Material and Methods.

The mouse is an animal well adapted to such a study, for we know from the investigations of Sobotta, Kirkham, and later of Mark and Long, that ovulation takes place without the necessity of copulation in from thirteen and three-quarter to twenty-eight and one-half hours (Mark and Long) after parturition. The sexes were kept together in suitable cages and the females examined frequently for signs of pregnancy. Whenever a female was found to be pregnant she was placed in a cage and examined each morning. As soon as a litter was found it was removed, and a record of the time made. It is from this time record that all the ages for the various eggs are figured.

Animals were killed at intervals varying from one and one half days to four days and nine hours after the finding of the litter. The body was immediately cut open and the ovaries with tube and uterus removed and placed in the fixing solution. For this purpose Zenker with acetic, and Carnoy's 6–3–1 solution, were found to be about equally good. For tube eggs strong Flemming proved excellent. The prepared sections were stained in Heidenhain's iron hæmatoxylin, Ehrlich's hæmatoxylin, or Flemming's Triple stain, usually with suitable counter stain.

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OBSERVATIONS.

Thirty-six hours after finding the litter, eggs are almost certain to be found in the upper part of the Fallopian tube. They always show the second maturation spindle and may or may not have the polar body attached. If present, the polar body has the chromosomes arranged in the form of a spindle (Fig. 1.) During the next twelve hours there is a change in the polar bodies. The chromatin of the spindle breaks down and arranges itself into one, two or many resting nuclei. The formation of two nuclei is well illustrated in Fig. 2. Here the vesicles have been formed but a number of chromosomes or chromatin bodies have not yet entirely lost their individuality. To one side of the polar body a small body, the first indication of a mitochondrial substance, may be seen. Fig. 3 shows a later stage in which the chromatin of the polar body has formed eight vesicle-like nuclei. Since eggs with three or more polar bodies are not infrequent (see Fig. 4), one is led to believe that the cytoplasm segments, forming itself around the various vesicles. Long and Mark ('11) find that the first polar cell often divides amitotically. They consider that this aids the degeneration and absorbtion of the polar bodies due to the increase in the exposed surfaces. Kirkham also, in work which has never been published, has observed that the first polar cell occasionally divides forming a number of fragments.

The achromatic fibers of the second maturation spindle disappear early, since in only a few cases have they been noted in eggs as old as forty-eight hours. The spindle stands out clear and distinct with the chromosomes in their natural relations but no signs of fibers can be observed.

Between forty-eight and seventy-two hours a renewal of activity begins in the unfertilized egg. Its effect is first apparent in the second maturation spindle, which may break down and form a single resting nucleus. Three eggs from a seventy-six hour mouse all have a single nucleus both in the eggs and in their polar bodies. More often however numerous vesicles instead of one result from the breaking down of the spindle. An examination of Fig. 5 will make it clear how this takes place. This egg is from a mouse in which one of the uterine horns was

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closed by a tumor, thus preventing access of the sperm to the eggs on that side. The mouse was killed sixty hours after finding the litter. In the tube on the occluded side, two eggs were found, one in the second maturation spindle stage, and the other showing the breaking down of the spindle. In the other horn fertilization had taken place and the eggs were in the four-cell stage. In the figure it will be noted that the chromatin of the spindle has for the most part gone into the formation of a circular vesicle, in which a number of chromosomes remain practically unaltered.

A later stage picturing the breaking down of the spindle is that of Fig. 6. Here the lobes are larger and have become separated from the central vesicle. All the chromatin is now found in the walls of the various vesicles, none remaining as chromosomes.

The loss of the achromatic fibers and the breaking down of the spindle is probably due, as suggested by Kingery ('13), to the degenerative changes appearing quite early in the cytoplasm. This is shown by the fact that the cytoplasm stains more deeply than in normal eggs. Numerous dark staining granules are seen, particularly near the periphery of the egg. These constitute the mitochondrial bodies.

Kingery ('13) is of the opinion that the spindle fibers of the second maturation spindle break and free the chromosomes, which then migrate into the cytoplasm and form numerous nuclear vesicles. In my material the achromatic fibers disappear early, and from Figs. 5 and 6 it would appear that the vesicles may be formed *in situ*, and not necessarily from chromosomes which have wandered from the place of the original spindle.

In Figs. 7, 8 and 9 a different process of spindle disintegration is seen. These three eggs are all from the same mouse and were located close together in the lowest portion of the tube very near the uterus. From Fig. 7, it is evident that while the spindle fibers have disappeared; the chromosomes, although contracted and hugged closely together, still retain the form of a second spindle. Several chromatic fragments have evidently broken off and are seen scattered in the cytoplasm. The succeeding stage may be noted in Fig. 8. The individual chromosomes

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have separated from their spindle position and lie scattered in the cytoplasm. One of the chromosomes shows the beginning of vesicle formation, which is seen completed in Fig. 9. Fig. 11 shows essentially the same condition. The polar body has divided and several small phagocytes may be seen just inside the zona. In Fig. 10 the chromatin is arranged around the periphery of the vesicle and not clumped to one side as in Fig. 9. One notices quite a similarity between these vesicles and those in process of formation in Fig. 6, and suggests that they may have been preceeded by such a stage.

After the formation of these numerous nuclear vesicles, the fragmentation of the cytoplasm takes place. Figs. 16 and 17, adjacent sections of a seventy-two hour egg, indicate that the fragmentation may take the form of a protoplasmic budding. The large cell shown in the figure has constricted off two fragments and is forming others. Adjoining sections show five additional fragments, making seven in all. Nuclear material is present in only two of the seven fragments. Of course one cannot say that all such fragments are constricted off from the egg, for there is always the possibility that some of them may have come from the polar bodies. It has been shown by Hartman ('16) in the opossum, and by Newman for the armadillo that normally one of the first activities of the unfertilized egg is the unburdening itself of the deutoplasmic material. This is done without assistance of any spindle or apparent nuclear influence. While it cannot be said that the fragments budded off in the mouse are exclusively of yolk material, the process is essentially a similar one.

Figs. 13, 14 and 15 are later stages in the fragmentation process. They represent what Kingery ('13) calls the "morula" stage. Figs. 14 and 15 represent two adjoining sections of the same "morula," which consists of about twenty-one cytoplasmic fragments.

We have seen how the second maturation spindle breaks down and forms either a single resting nucleus or numerous nuclei, and have traced the latter to the "morula" stage. When, on the breaking down of the spindle the egg is able to form a single or only a few resting nuclei, it would seem to indicate exceptional

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vitality and a close approximation of the normal condition. In such an egg the process of atresia would naturally be somewhat delayed. The egg with but one or two nuclei does not fragment immediately, and it is possible that such eggs may pass from the body, as suggested by Hartman ('16) for the unfertilized egg of the opossum. This possibility is well illustrated in Fig. 18, which shows an unfertilized egg one hundred and five hours after finding the litter. Only two nuclei are present. The mitochondrial bodies (*b*) can be distinguished from the nuclei by their peculiar crystalline appearance and elliptical shape. Although these bodies are found in most of the fragmented segments to be described later, they are smaller and stain less intensely in hæmatoxylin. They are surrounded by a light area which gives them the appearance of having shrunken from the cytoplasm. In Fig. 19 the mitochondrial bodies are seen to be smaller but more numerous. In the two upper fragments they did not cut cleanly but were pushed aside by the knife. This would seem to indicate that they were much more compact and dense than the surrounding cytoplasm.

Hartman ('16) finds a similar structure in the egg of the opossum, and describes it as a homogeneous, non-granular body staining pink or lavender in iron hæmatoxylin, and bordered by a light-colored band.

The multinucleated eggs soon break up into a number of fragments, often forming two or more parts very nearly of the same size and often appearing like normal cleavage stages, were it not for the presence of extra nuclei. Figs. 12, 13 and 14 show different stages in this process.

The ultimate fate of the fragmented egg will now be considered. Very early in the degenerative process, phagocytic cells are found inside the zona pellucida, between the cytoplasm and the zona. It is impossible to mistake these cells for fragments of either the egg or polar bodies for they appear almost like transparent vesicles with the deep staining nuclear material either scattered as granules or irregularly clumped in a way which immediately suggests their origin as polymorphonuclear leucocytes. Kingery described similar cells but thought them of follicular origin. The number of these phagocytic cells inside the zona varies,

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eight or ten being the largest number found. These cells probably aid in the disintegration of the periphery of the cell. This process may begin some time before fragmentation sets in, as is evidenced by a study of Fig. 9. Here the space at one end of the egg filled with dark granules indicates that the cytolysis of the cytoplasm has begun.

The zona persists in nearly all cases as far as the eggs have been followed. Fig. 6 shows one of the exceptions, for here the zona is gone and many dark staining fragments which may have come either from the polar body (which is now very small) or from the broken down zona, are seen surrounding the egg. Several phagocytic cells, one of which is figured, are to be seen among the fragments. Although the zona is present in almost all cases, a careful examination usually brings to light several breaks, and it is probably through these that the phagocytic cells are able to reach the egg.

Figs. 19 and 20 show the last stages in the disintegration and absorbtion of the fragments by the phagocytes. Some of the fragments appear as mere shells with only occasional granules, while the outer edge of others shows a honeycombed condition due to excessive vacuolization.

It is found that the unfertilized egg completes its passage through the Fallopian tube and enters the uterus about the end of the third day or almost at exactly the same time as given by Sobotta for the normal segmenting egg.

Smith ('17), basing ovulation as occurring twenty hours after parturition, finds that unfertilized eggs of the mouse enter the uterus about 76 hours after ovulation and are found in the last fold of the uterus at 88 hours.

The final dissolution of the fragmented egg evidently is completed early on the fourth day, since out of eleven mice older than four days, all showing corpora lutea in the ovaries, indicating that ovulation had taken place, in only three could eggs be found. A single egg was found in the uterus of a mouse one hundred and five hours after parturition (Fig. 18).

In not a single case has anything approaching a cleavage spindle as described in atresic ova by Spuler, Loeb or Newman, been observed, but it is not to be interpreted that they may

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not occur, because cleavage spindles are so short lived that the study of a much greater number of eggs would be necessary before coming to any such conclusion.

SUMMARY.

The cytological changes which the unfertilized egg of the mouse undergoes in the Fallopian tube and uterus are closely similar to those which occur in the eggs of atresic follicles in the ovary.

The processes are considered degenerative and have only a superficial resemblance to parthenogenetic development.

The breaking down of the second maturation spindle of the unfertilized egg usually results in the formation of several or many nuclei. Rarely a single nucleus is formed, in which case the egg does not fragment rapidly and may pass from the uterus before the degenerative processes are complete.

The eggs with many nuclei divide into numerous cells, of which some are provided with degenerating nuclei. These are further disintegrated and absorbed by phagocytic cells, which make their way into the egg probably through breaks in the zona. The phagocytes from their appearance are polymorphonuclear leucocytes, and they evidently act on the cytoplasm causing a vacuolization of its outer portions and a later crumbling of its periphery; the end being a complete disintegration of the egg and its absorption by the phagocytes.

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EXPLANATION OF PLATES.

All the drawings are from sections of unfertilized eggs in the Fallopian tube or uterus. They were made with the aid of a camera lucida, and with the exception of Fig. 5 are reproduced at a magnification of 900 diameters.

Abbreviations.

a, zona. *b*, mitochondrial body. *c*, phagocytes. *d*, vacuole. *e*, degenerating protoplasm.

Plate I.

Fig. 1. (48 hours.) Egg with the second maturation spindle. Polar body shows chromosomes arranged on the spindle fibers. Eggs of this type, showing no changes are found in the tube up to forty-eight hours after parturition.

Fig. 2. (56 hours.) In this egg the spindle is present but the chromosomes seem to have clumped together more than in Fig. 1. The spindle of the polar body has disappeared and in its place are seen two resting nuclei.

Fig. 3. Polar body (60 hours) from same mouse as Fig. 5 showing many resting nuclei formed from the breaking down of the polar spindle or by the chromatin fragments often observed in the polar body.

Fig. 4. (48 hours.) Showing the fragmentation of the polar body. Here the first polar body has divided once and one of the parts has redivided. The spindle cut somewhat obliquely is one of few showing good achromatic fibers. These fibers seem to end in a centrosome-like body.

Fig. 5. (60 hours.) Chromosomes of the second maturation spindle breaking down and forming resting nuclei. Vesicular lobes are seen extending out from the main vesicle. Quite a number of the chromosomes have not yet entered into the process and are seen in the lobes of the forming nuclei. This mouse had a tumor in one of the uteri which prevented fertilization, while on the other side, eggs in the tube had reached the four cell stage. ($\times$ 1,800.)

Fig. 6. (78 hours.) A later stage of vesicle formation from the maturation spindle. Chromatin all in the vesicles, no chromosomes as such. This was one of the few eggs in which the zona was missing. Many dark staining granules surrounded the egg and in among them several phagocytes. One phagocyte shown at left of polar body, which is much smaller than normal, suggesting that the phagocytes have been acting upon it and probably upon the zona also.

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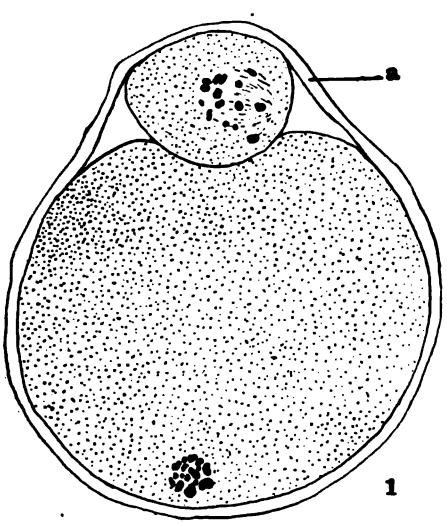

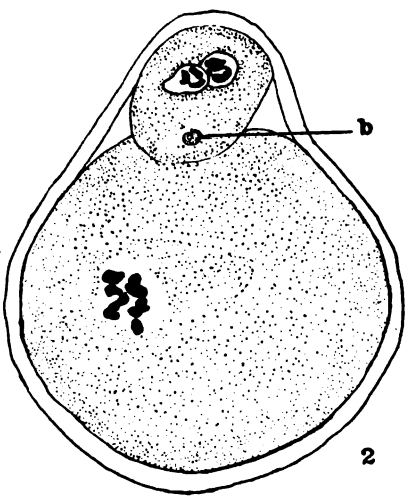

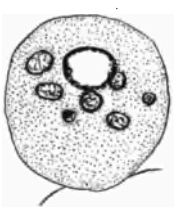

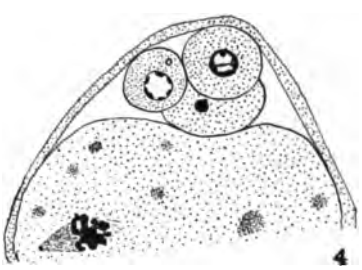

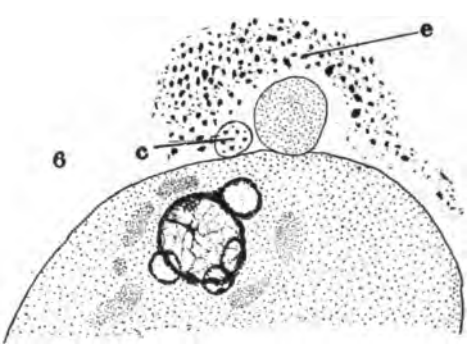

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PLATE II.

FIG. 7. (72 hours.) Spindle fibers missing, chromosomes of spindle hugged closely together. Several parts separated and out in the cytoplasm.

FIG. 8. (72 hours.) A later stage of the above. Chromosomes scattered in the cytoplasm. One showing the beginning of vesicle formation. Several phagocytes are present inside the zona. Cytoplasm near open end shows beginning of disintegration.

FIG. 9. (72 hours.) Completion of vesicle formation begun in Fig. 8. The chromatic material clumped to one side of the vesicle. Quite a few small particles of disintegrated cytoplasm may be seen inside the zona indicating the activity of the phagocytes.

FIG. 10. (72 hours.) Reconstruction showing ring-like formation of nuclear material. Possibly a succeeding stage to Fig. 6. A phagocyte cell is noticed just outside the zona, which is very faintly stained.

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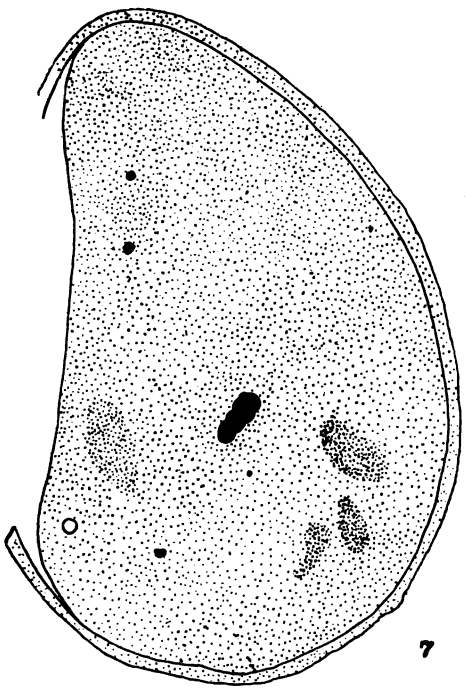

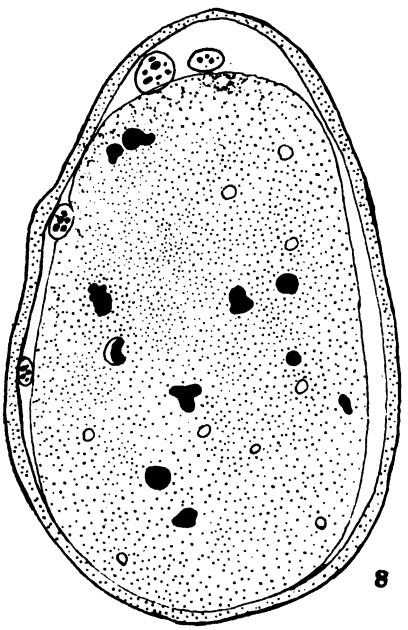

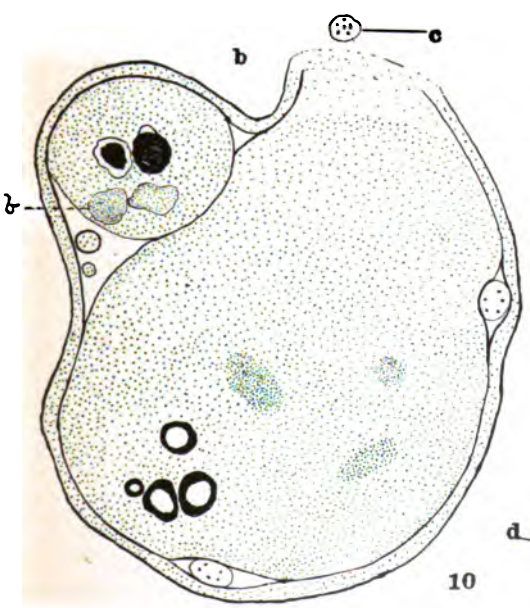

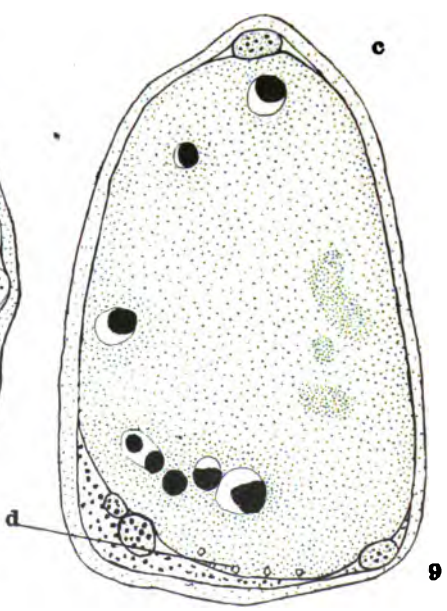

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PLATE III.

FIG. 11. (72 hours.) Egg showing four resting nuclei, and a number of mitochondrial bodies. In addition a divided polar body and two or more smaller structures which are probably phagocytic cells.

FIG. 12. (72 hours.) Fragmented egg looking very much like a normal two-cell stage, except that each blastomere has two or three nuclei. The nuclei contain nucleoli and appear perfectly normal.

FIG. 13. (81 hours.) A later stage of fragmentation. A large resting nucleus is seen in one of the larger segments, while other fragments have none. In two of the cells the mitochondrial body is quite conspicuous.

FIG. 14. Egg of uncertain age, consisting of twenty-one fragments. Numerous phagocytic cells are seen in and about the fragments. Zona entirely missing.

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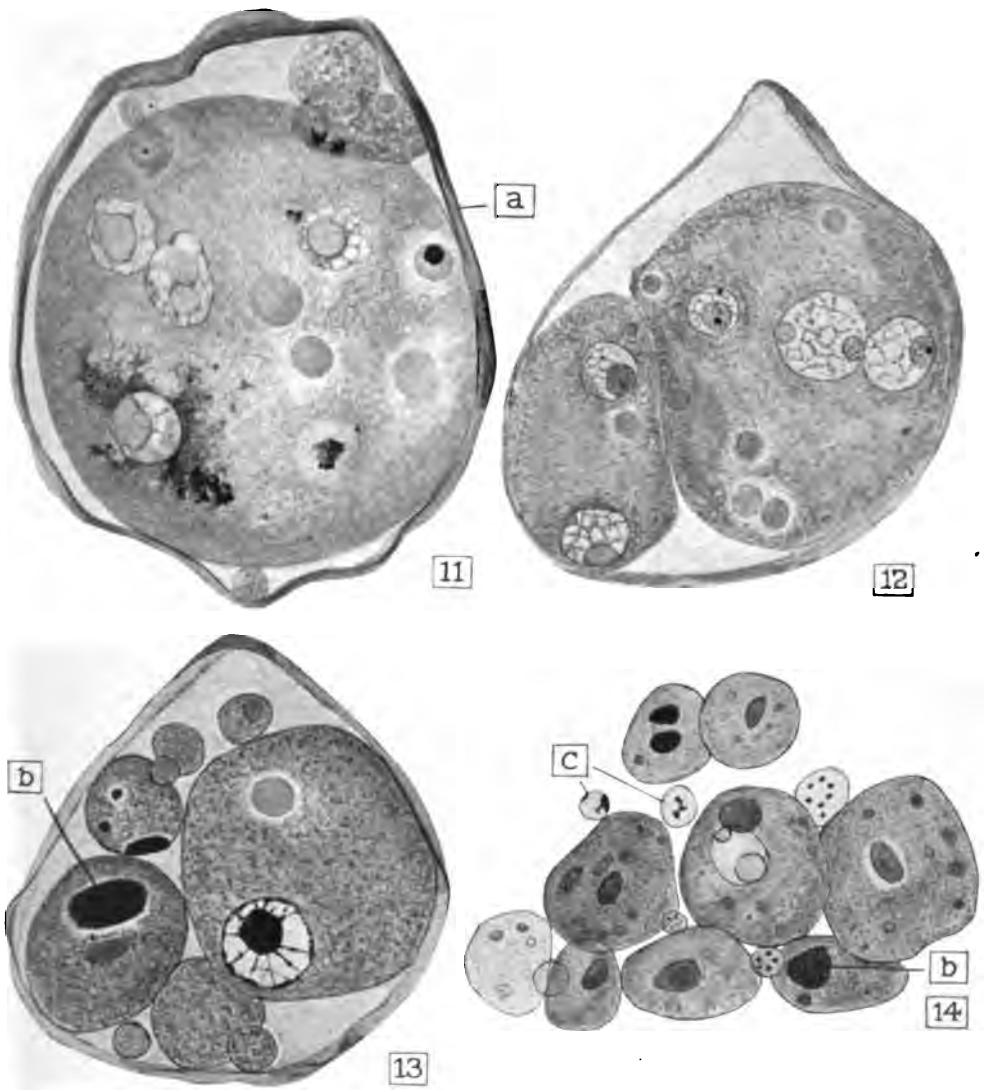

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PLATE IV.

FIG. 15. Drawing of adjacent section to that shown in Fig. 14.

FIG. 16. (72 hours.) Egg showing the formation of protoplasmic fragments independent of any spindle. These are later pinched off and form small segments inside the zona.

FIG. 17. Drawing of adjacent section to that shown in Fig. 16. A study of all the sections of this egg shows that seven fragments have been pinched off, and that of these only two appear to contain nuclear material.

FIG. 18. (105 hours.) Egg is without zona, contains two nuclei and two immensely large refractive mitochondrial bodies. The polar body still remains attached to the egg.

FIG. 19. An 81-hour egg showing phagocytic effect. In two upper cells the mitochondrial bodies have been pulled out of the cytoplasm towards the left by the knife, leaving clear spaces where they originally were, indicating the solid character of these structures. Many vacuoles are seen around the border of the fragments. Between the cells dark stained particles of degenerated cytoplasm is being absorbed by the phagocytes.

FIG. 20. (81 hours.) A later stage than Fig. 19. In one of the fragments hardly any content is to be seen except a few scattered granules.

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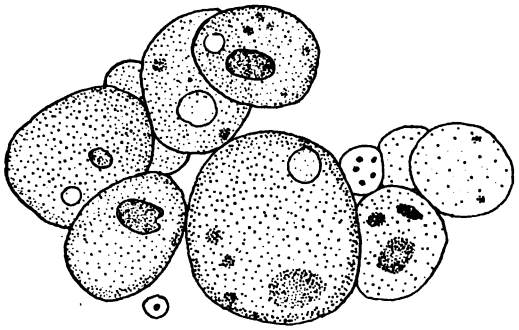

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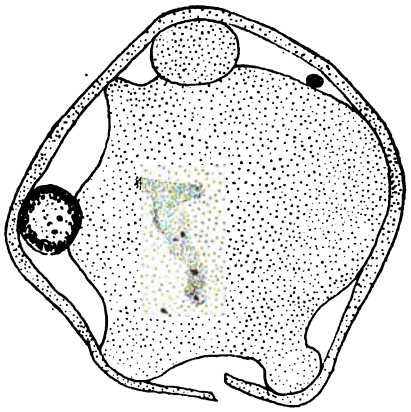

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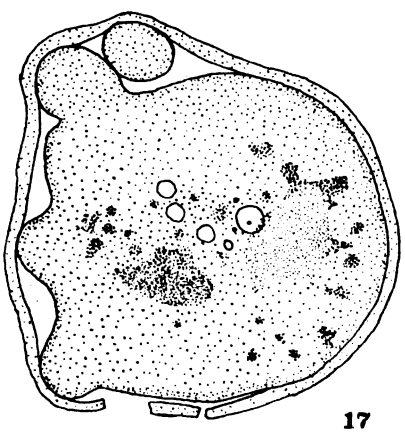

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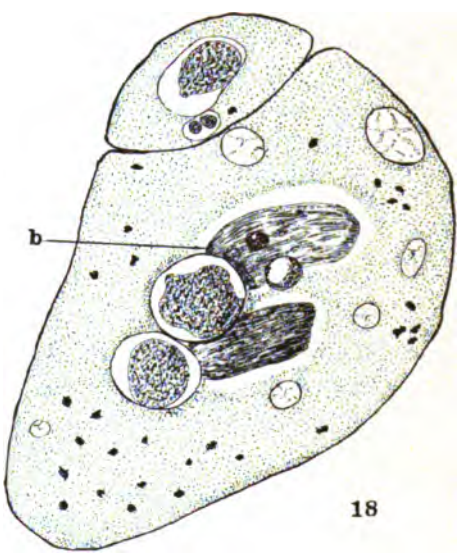

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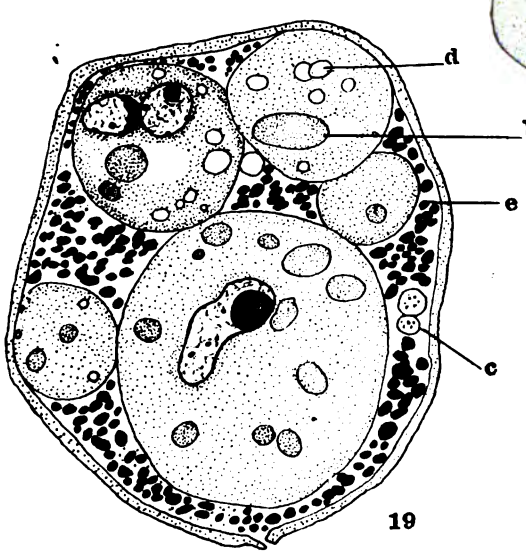

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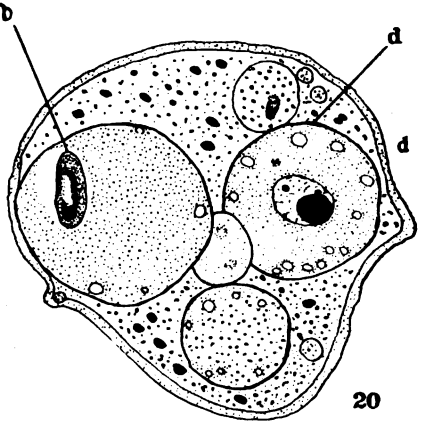

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H. W. CHARLTON.

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OBSERVATIONS ON THE STRUCTURE OF DOUBLE MONSTERS IN THE EARTHWORM.[1]

ROXIE A. WEBER.

I. Introduction.

The work which has thus far been done on double monsters in the terricolous group of annelids is quite limited, since in only a few species, *Lumbricus trapezoides*, *Lumbricus terrestris*, *Allolobophora fœtida* and *Allolobophora trapezoides*, has this condition been observed. The first of these forms is described by Kleinenberg, the remaining three by Vedjovsky. These two investigators agree neither as to the method of union nor as to the cause of this phenomenon. It is not the object of this paper to give the factors influencing such monster formations, but merely to describe the manner in which the members are joined.

Material and Technique.—The material studied consists of twelve double embryos, the shortest member of which is at least 60 segments in length, the longest 125, one monster in which one individual consists of 16 the other 17 segments, 19 double gastrulæ and two embryos showing a bud on one side of the blastopore, and one case of an egg consisting of two distinct hemispheres connected by a band of large cells. The entire supply was obtained from Professor Patterson, of this University.

The fixing fluids employed were Meves, Gilson, and Bouin, all of which gave only fairly good results. Ehrlich's hæmatoxylin was used as a stain.

The species studied is *Helodrilus caliginosus trapezoides*, according to Professor F. Smith's identification.

Review of the Literature.—Before describing the individuals studied, it will perhaps be well to give a brief review of the results of the two authors above mentioned.

Kleinenberg found that the number of eggs in the capsules of *L. trapezoides* varies from three to eight of which usually one,

[1] Contributions from the Zoölogical Laboratory of The University of Texas, No. 139.

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though sometimes two or three, produces an embryo. The remaining ones, not becoming fertilized, disintegrate completely.

By a series of somewhat irregular cleavages the egg develops into a germinal bladder, one layer in thickness (Pl. IX., Fig. 2, Kleinenberg). Thereupon a two-layered condition begins to appear, one side of the cell mass becoming differentiated in advance of the other. At this pole all the cells divide rapidly except two, which become pushed in and covered over by the small blastomeres. From these two cells (mesoblasts) are derived the first rudiments of entoderm and mesoderm (Pl. IX., Fig. 4).

While this elongation is taking place a transverse furrow appears midway between the two ends extending almost entirely around, leaving the two hemispheres connected by only a few enlarged ectodermal cells.

When this stage has been reached the cells of the other pole begin to undergo the same changes and finally we have formed from each half an embryo joined in varying degrees to the other by a band of ectodermal cells. The separation is eventually effected by a series of rotations which usually result in the breaking of the uniting cord. When for one reason or another this is not accomplished we have true monstrosities in all degrees of coalescence.

According to Kleinenberg the union never extends to the internal organs but is confined to the external epithelium of the body wall.

The monsters formed in this manner are of the same or nearly the same size. There is however still another type of abnormality (Pl. IX., Fig. 10), *e. g.*, those showing bud formation. Kleinenberg explains their production as being due to a very unequal development of the two halves of the above mentioned cell mass.

In all his work he has found only a few cases in which two individuals did not emerge from one capsule and in those few exceptions rudiments of a second were usually found. For this reason he concludes that each egg produces two individuals normally.

Vedjovsky on the other hand holds this condition to be ab-

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normal. He explains the formation of double embryos as being due to what he terms *Doppelfurchung*. The egg elongates in the direction of the animal pole and subsequently divides into two blastomeres either of equal or unequal size. They are designated as A and a. From each of these cells there is produced a quartet, one lying upon the other giving the appearance of micro- and macromeres in the cleavage of *Crepidula* or the eight cell stage of *Synapta*, depending on the proportionate sizes of A and a. Each of the quartets finally gives rise to an individual which remains attached to its companion. If the a cell is smaller than A, there is produced one well developed individual and a bud.

This author has found examples of monster formation in *Lumbricus terrestris*, *Allolobophora fœtida* and in *Allolobophora trapezoides*. In the first group he found two cases in both of which the two individuals were joined on the dorsal side through one segment. Only one monster was found among several hundred embryos of the second group. In the third group a large number was found which the author describes under three divisions.

A. Those monsters in which the individuals are united on the ventral side along the entire length of the body (Pl. 19, Fig. 14).

B. The cases in which the members are joined on the dorsal side (Plate 21, Fig. 7).

C. The double monsters in which the individuals are fused end to end. (*a*) Those in which both members are of similar length (Pl. 21, Fig. 9). (*b*) Those cases in which one member is rudimentary (Pl. 19, Fig. 12).

II. Observations.

Even though these two workers differ so markedly in their conclusions it will be noted from the following that the observations made by the writer are similar in many respects to those made by both of these men.

In this study of the monsters of *Helodrilus caliginosus trapezoides* it has been found that the greatest variety exists in the structure of the nervous system. There is in every case a complete union of the five layers of the body wall, and the

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digestive tract is either fused or not fused, but the nervous system is modified in a variety of ways. The manner and extent of union of these individuals and their organ systems are best described in the following groups.

1. Those double monsters in which the union is dorsal. This group may again be subdivided into—

(*a*) Those in which the union extends to the alimentary tracts. Of these there are two examples, Nos. 95 and 171, Plate I. From Figs. 1 and 2, Plate I., it can be seen that the fusion in No. 171 is through one segment only, while in No. 95 it extends through five. A description of sections through this monster will serve to show the general relationship of the organ systems in the members of this group. Fig. 3, Plate I., is a section through the anterior portion showing the condition of the nervous system. It will be seen that there is almost a continuous band of nerve tissue extending around the pharynx. A study of the neighboring sections shows that this band is really complete. It will also show that the portion marked (*b*) in the figure enlarges into a bilobed brain and that a similar structure exists on the opposite side of the pharynx. At the same time there is also to be found at each end of the greatly elongated pharynx another set of ganglia. It would be impossible here to determine which are brain and which are ventral cord ganglia were it not for the presence of setæ (*s*). This at once leads to the conclusion that the ganglia at the end of the pharynx must be those of the ventral cord. This inference is further proven by a study of sections through a more posterior region, as shown in Fig. 4, Plate I. Here are no longer to be found the ganglia on either side of the pharynx but only those at the opposite ends. This section too shows the setæ in normal relationship with the ventral cord and the nephridæ in their natural position. They are shown only on one side but those of the other half can be seen in the next section.

It will be noted that the single pharynx is greatly elongated from end to end of the monster. Throughout the fused part it remains as one cavity dividing into two parts only when the point of separation of the two members is reached, one passing into each individual. Surrounding the pharyngeal cavity there

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is a great mass of muscular tissue attached by thin strands to the body wall. This condition is exactly similar to that found in single individuals.

The sub-intestinal blood vessels are clearly visible (*v.b.v.*) in their normal position between the alimentary tract and the nerve cord. On each side of the muscular pharynx there lies a more or less regular vessel (*D.B.V.*) which when traced to the point of separation of the two members will be found to approach the center and then one passes into one individual, the other into the other, the vessel on the left side passing into the upper half, the one on the right into the lower half.

Though the other monster is formed by a coalescence of the two members through one segment only, that union is complete in every respect from body wall to pharynx. Fig. 5 is a longitudinal section through No. 171 showing a portion of one of the brain ganglia together with a small part of the nerve cord of each of the individuals. A study of the successive sections will show that the pharynx arises from a flattened portion which is lined with the same kind of epithelial cells as the rest of the digestive tract and which lies between the two members as indicated in Fig. 2, Plate I. (*m*). The ventral cords are found to be connected to the cerebral ganglia, of which there are two sets, one on each side of the mouth opening, by means of commissures as in No. 95.

The setæ, not shown in this figure, and the nephridæ lie along the same side of the body as the nerve cord.

(*b*) Those in which the alimentary tracts have not become united. Nos. 91, 92, 142 and 173 shown in Plate II as Figs. 6, 7, 8 and 9 illustrate this group. It will be noted that though only one definite pair of segments appear to be fused in the first three cases, there is in each instance an irregular mass of tissue between the separated edges of the fused pair of segments. Whether these masses are modified segments has not been determined.

In all four instances the nerve cords are formed on the side opposite the line of fusion. These cords are joined to the bilobed brains in a manner very similar to that found in No. 95 (Fig. 3). It is to be noted that the union of these individuals

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is the same in kind and extent as in the group already described except for the digestive tract. In every one of the four there is distinguishable a separate alimentary tract for each member beginning with the mouths. These structures lie very near the nerve cord and are bounded on the inner side by a mass of muscles similar to those which usually lie on either side of the elongated fused pharynx.

The fixation of the blood vessels was such that they could not be studied.

The members of these groups to which belong half the specimens studied, have undoubtedly been fused along the dorsal side. The relative positions of setæ, nephridæ, and nerve cord are positive proof thereof. Vedjovsky gives a number of figures, very similar to Figs. 3 and 4, in his paper but he explains the union as being along the ventral side. It is impossible to disprove this conclusion for the two members were joined along their entire lengths. In the cases described in this paper, the union was never through more than five segments If, then, the coalescence had been other than dorsal, it is very probable that the nerve cord together with the setæ and excretory organs would have swung around to their natural position in the separated portions of the two individuals. This, however, is not the case. Therefore, the conclusion must be drawn that at least in these instances the union is no other than dorsal.

It is very probable that the one case described by Vedjovsky is also one of dorsal instead of ventral union, and that the cerebral ganglia are composed of half from each member instead of this being the case for the ventral cords, as that author supposes. If those individuals in which the digestive tract was separate had not been found it would not be possible to make this statement for it might be argued that each side of the pharynx was formed from the alimentary tract of one member. But since those cases have been found it can be easily seen that a joining of these structures would have resulted in forming a greatly elongated organ similar to that shown in Vedjovsky's figures. Furthermore, if the union in these forms had been ventral, the pharynges would certainly not have been found in the positions in which they lie, but would have been much nearer together in the central portion and side by side instead of end to end.

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2. Those cases in which the union is latero-dorsal. Thus far only one example has been found of this group, individual No. 70 shown in Fig. 10, Plate II. Figs. 11 and 12, Plate II., show sections through the joined region which in this case extends through three segments. The first is very much more anterior than the second as is indicated by the presence of the cerebral ganglia (*bg*) of which there is in this monster only one set. The commissures extending from it can be seen in the same figure in which is also present the beginning of one of the nerve cords (*nc*). A study of the next few sections shows the connection of the brain to the two cords and also a connecting band between these two structures. In this manner the single greatly elongated alimentary tract, which in this case is the result of a union, is completely surrounded by nerve tissue. Fig. 12 gives a section through a more posterior region showing the relative positions of the ventral cords.

In no part of the joined portion can there be found less than eight pairs of setæ indicating that the union does not extend beyond the sides of the individuals.

The nephridæ also are in their normal number and relationship to the ventral cord.

3. This is the group in which the union is end to end and in which the cerebral ganglia are to be found on the opposite side of the digestive tract from the ventral cord. It can be seen from an inspection of Figs. 13, 14 and 15, external drawings of Nos. 90, 93 and 2, that the extent of union is limited to an unusually small area. Sections show that the union is across the dorsal side, for the ventral cords lie on the sides opposite that area, while the cerebral ganglia lie on the same side.

The digestive tracts have a common origin in each of the three monsters arising from a single mouth opening from which the pharynx passes into each individual. There is no noticeable difference in the structure of any of the fused organs of these forms as compared with those of No. 95 except in the nervous system.

A study of No. 90 will show a condition of that system somewhat different from any so far described. The ventral cord of each individual lies on the side opposite the union while the

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brains lie on the same side and are connected to the cords by means of commissures, a condition very similar to that found in normal single individuals. The brains however in this instance are joined to each other. In one of the individuals the commissural connection between brain and cord can be seen beyond the point of separation, but in the other member the two ganglia of the brain seem to be separated and joined separately to the cerebral ganglia of the first member.

In No. 93, shown in Fig. 14, there is present only one set of cerebral ganglia greatly elongated. It lies on that side of the pharynx opposite the cords and is joined to that structure in one member by two commissures, to that in the other by only one.

A study of sections through No. 2 reveals a condition similar to that in No. 90. There are two distinct sets of cerebral ganglia, one joined to each nerve cord as in normal individuals and a connection between the two brains much as between the two cords in No. 70.

4. That group of double monsters in which the union between the two individuals has been side to side with both mouth openings on the same side, illustrated by Nos. 67 and 1.

In No. 1 the union is through one segment only as indicated in Fig. 16, Plate III. There is a single mouth opening lined with the columnar epithelial cells, from which the digestive tract of each individual passes inward. There can be seen on one side of the pharynx in a transverse section a single set of cerebral ganglia which is joined to the ventral cords by commissural strands extending over the pharynx. There are no such structures to be found on the under side of the alimentary tract. On the opposite side of the section there can be traced a connection between the cords themselves. Fig. 17, Plate III., is a section through the united portion of No. 1 showing the single pharynx, cerebral and ventral ganglia and a portion of the strand of nerve tissue connecting the two cords.

A study of sections through No. 67, shown in Fig. 18, Plate III., will show that the two mouth openings have been joined into one, and that the ventral cords lie on one side of the common pharynx and the cerebral ganglia on the other. There is only one set of brain ganglia to be found in this monster, but,

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unlike all other cases, there are three distinct commissural strands joining it to the nerve cords, one extending over the top of the pharynx, the other two below the point of union of the two digestive tracts forming a triangle with each other. Fig. 19, Plate III., is a cross section of these last mentioned strands and their connection to the two cords. As in No. 70 there is in this monster also a limited connection between the two cords.

5. Cases in which the two individuals are extremely unequal in size, *e. g.*, bud formation. Thus far there have been found two such cases, a section of one being shown in Fig. 20, Plate III.

III. Discussion.

It will be remembered that reference was made to the difference in view concerning the origin of double monsters in an earlier part of this paper. Vedjovsky maintains that the formation of two embryos from one egg is abnormal, whereas Kleinenberg maintains the opposite. A difference of this kind may be explained on the basis of the difference in the forms worked on by these two investigators. This paper is a study of a form very similar, in its mode of development, to that described by Kleinenberg. It has been found that it is quite common, not only for monsters to appear in this group, but also for two individuals to emerge from one capsule. Out of the 184 cocoons opened 57 contained only one individual, 101 two, and 25 eggs in various cleavage stages and 1 four embryos. Thirty-five of the 101 cases were in the form of monsters. Six cases have been noted in which two or more eggs were present but in four of these only one was undergoing development while the others were in various stages of degeneration. One egg was found which had just reached a stage in development sufficiently far advanced to show the division into two hemispheres and the connecting band of larger cells very similar to Kleinenberg's Fig. 6. Three others were found which had begun to elongate in a manner very similar to the above.

It would certainly be safe to conclude that it is a common occurrence for one egg of this species to give rise to two individuals. If this were not the case it would seem rather unusual to find so large a number of cases in which two and only two

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individuals emerge from one capsule when the number of eggs found in one capsule varies ordinarily from three to eight. The elongation of those eggs sufficiently far advanced in development to show this phenomenon is still further indication of this same thing.

Kleinenberg attributes the cause of this double embryo formation to the entrance of two sperm into a single egg setting up two points of activity. In the light of more modern discoveries this explanation no longer holds good. Vedjovsky suggests the possibility of temperature and moisture changes, and exposure to air bearing their influence on the egg and causing its abnormal development. Until further studies are made along this line it will be impossible to state any definite causes of monster formation.

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Explanation of Plate I.

Helodrilus caliginosus trapezoides.

1. No. 95, showing union through five segments.

2. No. 171, showing union through one segment.

3. Section through the anterior portion of No. 95, showing the pharynx fused and the extension of the nerve tissue almost entirely around the pharynx. *b*, cerebral ganglia; *s*, setæ.

4. A section through a more posterior region of the same monster. *Vg*, ventral ganglia; *DBv*, dorsal blood vessel; *VBv*, ventral blood vessel; *n*, nephridium; *s*, seta.

5. A longitudinal section through No. 171, showing a portion of the cerebral ganglia *b*, and also parts of the ventral cords.

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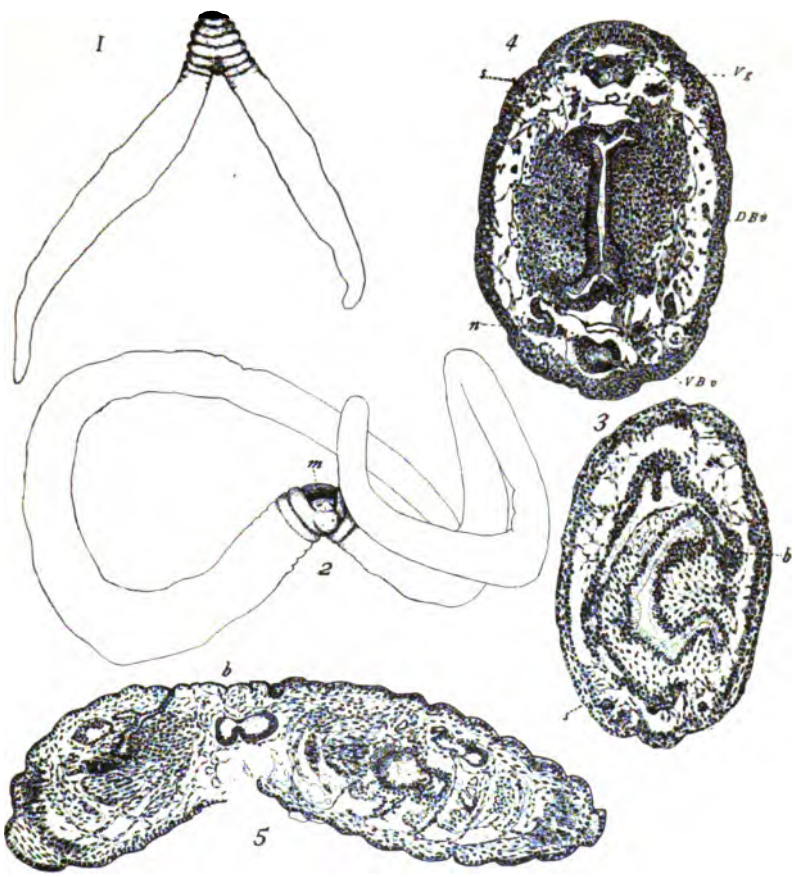

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Explanation of Plate II.

Helodrilus caliginosus trapezoides.

6. No. 91.
7. No. 92.
8. No. 172.

} Fused through one segment only with irregular masses between the separate edges of the united segment.

9. No. 173, united through four segments.

10. No. 70, united through four segments.

11. A section through the anterior portion of No. 70, showing a single set of cerebral ganglia (*bg*) and one of the ventral ganglia (*nc*).

12. A more posterior section of the same monster showing two ventral ganglia.

13*a* and *b*. No. 90.
14. No. 93.
15. No. 2.

} All show very limited connection.

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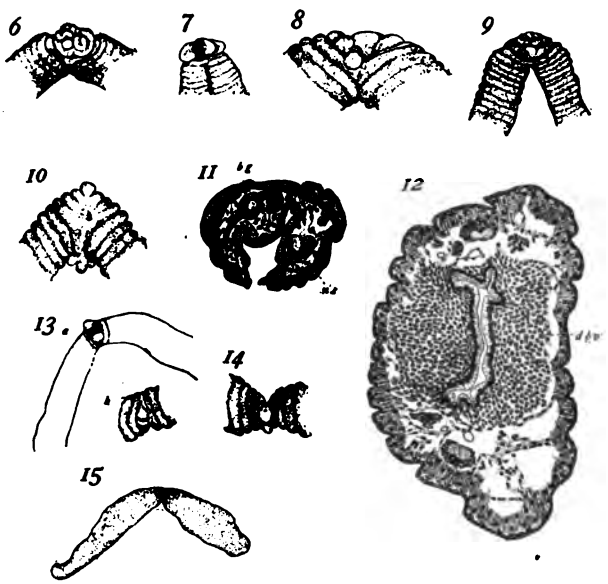

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Explanation of Plate III.

Helodrilus caliginosus trapezoides.

16. No. 1 viewed from the side of the mouth opening.

17. A section through No. 1 horizontal to the mouth opening, showing the cerebral ganglia on one side of the pharynx and the cord tissue on the opposite side.

18. No. 67, united through two segments.

19. A transverse section through the posterior part of the united portion showing the cerebral ganglia joined to the ventral ganglia (*vg*) by commissures.

20. A section through a gastrula stage showing bud formation.

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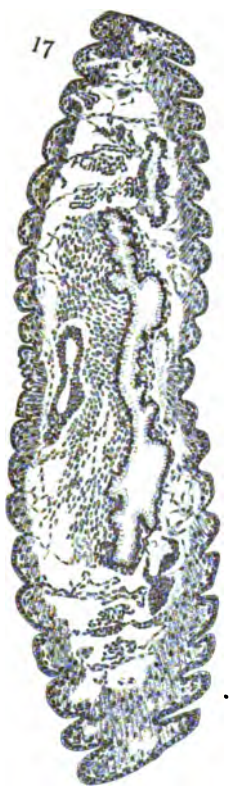

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IODINE AS A PARTHENOGENETIC AGENT.

ALVALYN E. WOODWARD AND FLORENCE S. HAGUE.

In the course of some experiments with *Arbacia* eggs at Woods Hole last summer, it was noted that, if a little iodine was added to the eggs in sea-water, regular fertilization membranes appeared on a large number. This led to an attempt to find the optimum conditions for the use of this reagent in parthenogenesis.

The stock solution used was made by dissolving iodine crystals in sea-water to the point of saturation. This stock solution was diluted with sea-water as shown in the tables.

Some of the females were stimulated to shed their eggs into watch glasses. In other cases, the ovaries were removed and shaken in sea-water to loosen them. Whichever method was followed, the eggs were washed and allowed to settle. The supernatant water was poured off, the eggs evenly mixed in fresh sea-water, and 2 c.c. of the mixture put into each of a series of watch glasses. To each of these an equal volume of iodine solution was added, for a given length of time. Some of the eggs were then put into finger-bowls containing about 50 c.c. of sea-water. Others were treated for 20 minutes with hypertonic sea-water (50 c.c. sea-water + 8 c.c. 2.5 N NaCl). At the end of that time, the solution was drawn off and the eggs transferred to finger bowls of sea-water. With each lot of eggs, three controls were made, one fertilized with sperm, one uninseminated, and one uninseminated but treated with hypertonic sea-water

Table I. shows the percentage of membranes and cleavages produced by varying the strength of the solution and the length of exposure. To obtain these averages, over two hundred eggs were counted in each case.

It will be noted that there is great variability in the results with different batches of eggs. This may be explainable from the fact that the experiments were carried on during the last two weeks of August and the first week of September, a period

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TABLE I.

PERCENTAGE OF CLEAVAGES AND MEMBRANES OBTAINED BY TREATING ARBACIA EGGS WITH IODINE, OR IODINE FOLLOWED BY HYPERTONIC SEA-WATER.

Time. Min.	Sat. Iodine Solution.		$\frac{1}{2}$ Sat. Iodine Solution.		$\frac{1}{4}$ Sat. Iodine Solution.		$\frac{1}{8}$ Sat. Iodine Solution.		$\frac{1}{16}$ Sat. Iodine Solution.		$\frac{1}{32}$ Sat. Iodine Solution.		$\frac{1}{64}$ Sat. Iodine Solution.		Controls.			Remarks.
		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.	Fertil-ized.	Not Fertilized. In Sea-water.	Not Fertilized. In Hyper-tonic.	
2.5			14	24											69	3	5	Shed
			14	22											64	2	2	Shaken
	2	7	17	16											68	4	2	Shaken
					26	27	16	16							41	1	4	
					16	32	11	39							69	3	11	
									12	13	12	14			85	7	9	
									5	8	4	12			83	7	9	
5					16	12	14	39	6	16	3	3			44	2	2	Shed
					18	21	30	22	17	17	5	15			32	1	2	Shed
					3	15	20	22	20	23	9	10			83	1	4	Shaken
			1	1	21	2	20	29	14	23					82	5	6	Both
							12	32							3	1	1	Shed
							19	20							94	5	6	Shaken
							16	27							94	5	6	Shaken
									8	21					66	3	5	Both
			20	17											69	3	5	Shed
			19	7											64	2	2	Shaken
	1	1	18	9											68	4	2	Shaken
					49	49	11	5							41	1	4	
					23	37	26	43							69	3	11	
									10	13	9	4			85	7	9	
									2	13	5	10			83	7	9	

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TABLE I.—*Continued.*

Time, Min.	Sat. Iodine Solution.		½ Sat. Iodine Solution.		¼ Sat. Iodine Solution.		⅛ Sat. Iodine Solution.		1/16 Sat. Iodine Solution.		1/32 Sat. Iodine Solution.		1/64 Sat. Iodine Solution.		Controls.			Remarks.
		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.		+ Hyper-tonic.	Fertil-ized.	Not Fertilized. In Sea-water.	Not Fertilized. In Hyper-tonic.	
7.5			5	21	29	20	16	13	1	2					13	0	1	Shed
			35	37	28	28	41	31	8	18					70	4	10	Shaken
							21	47							3	1	1	Shed
							30	22							94	5	6	Shaken
							22	27							94	5	6	Shaken
									9	26					66	3	5	Shed
			15	13											69	3	5	Shed
			11	10											64	2	2	Shaken
	7	4	23	16											68	4	2	Shaken
					34	24	24	27							41	1	4	
					25	36	28	27							69	3	11	
10			13	7	23	20	21	19	11	16					88	1	5	Shed
			0	15	30	26	25	19	8	21					51	0	3	Shed
			3	9	37	25	13	33	7	17					88	1	11	Shaken
			3	15	22	16	14	18	11	6					13	0	1	Shed
			28	23	19	23	11	21	23	32					70	4	10	Shaken
			1	1	1	8	26	31	28	28					82	5	6	Both
							45	56							3	1	1	Shed
							19	24							94	5	6	Shaken
							26	37							94	5	6	Shaken
									16	24					66	3	5	Both
			8	5											69	3	5	Shed
			7	14											64	2	2	Shaken
	3	3	11	17											68	4	2	Shaken

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TABLE I.—*Continued.*

Time, Min	Sat. Iodine Solution.	+ Hyper-tonic.	½ Sat. Iodine Solution.	+ Hyper-tonic.	¼ Sat. Iodine Solution.	+ Hyper-tonic.	⅛ Sat. Iodine Solution.	+ Hyper-tonic.	1/16 Sat. Iodine Solution.	+ Hyper-tonic.	1/32 Sat. Iodine Solution.	+ Hyper-tonic.	1/64 Sat. Iodine Solution.	+ Hyper-tonic.	Controls. Fertilized.	Not Fertilized. In Sea-water.	Not Fertilized. In Hyper-tonic.	Remarks.
10					32	44	18	14							41	1	4	
					26	39	18	44							69	3	11	
									15	12	4	15			85	7	9	
									6	17	4	10			83	7	9	
12.5							8	28							3	1	1	Shed
							13	18							94	5	6	Shaken
							23	24							94	5	6	Shaken
									16	26					66	3	5	Both
			12	6											69	3	5	Shed
			7	19											64	2	2	Shaken
	11	3	8	16											68	4	2	Shaken
					18	20	19	46							69	3	11	
15							15	33							3	1	1	Shed
							16	22							94	5	6	Shaken
							17	41							94	5	6	Shaken
									21	35					66	3	5	Both
					24	42	29	29							69	3	11	
									9	11	10	12			85	7	9	
					21	27	7	13					2	5	92	1	2	Shaken
20									15	17	14	15			85	7	9	
					21	24	9	5					2	8	92	1	2	Shaken
25									15	20	8	16			85	7	9	

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near the close of the breeding season, when the eggs vary greatly in their fertilizing power and their sensitiveness to reagents. It is probable that the strength found best for this season will prove too great for the more sensitive eggs in the height of the season—July, for instance.

Table II. is a summary of Table I., giving the average number

TABLE II.

SUMMARY OF TABLE I.

Time, Min.	Sat. Iodine Solution.		$\frac{1}{2}$ Sat. Iodine Solution.		$\frac{1}{3}$ Sat. Iodine Solution.		$\frac{1}{4}$ Sat. Iodine Solution.		$\frac{1}{6}$ Sat. Iodine Solution.		$\frac{1}{12}$ Sat. Iodine Solution.		$\frac{1}{16}$ Sat. Iodine Solution.	
		+ Hyp.		+ Hyp.		+ Hyp.		+ Hyp.		+ Hyp.		+ Hyp.		+ Hyp.
2.5	2	7	15	21	21	30	14	28	9	11	8	13		
5	*1*	*1*	15	9	22	23	19	27	11	18	6	8		
7.5	7	*4*	18	19	29	27	26	28	6	15				
10	*3*	*3*	6	12	24	25	20	26	14	19	4	13		
12.5	*11*	*3*	9	14	*18*	*20*	16	29	*16*	*26*				
15					23	35	17	28	15	23	*10*	*12*	2	*5*
20					*21*	*24*	*9*	*5*	*15*	*17*	*14*	*15*	2	*8*
25									*15*	*20*	*8*	*16*		

of cleavages plus membranes obtained by each method of treatment. A number in italics is the result of one experiment, rather than the average of several. There is a remarkable uniformity in the percentages obtained by treating the eggs for varying periods of time. For instance, whether exposed for two and one half or fifteen minutes, or for some intermediate period, to one eighth saturated iodine, the eggs gave percentages between 26 and 28. The indication is that the iodine enters the egg immediately and affects it immediately to its full extent. One would expect this result if it acted by combining chemically with something in the egg. While length of exposure has no effect, varying the strength of iodine used causes great variation in the result. This indicates a secondary, non-initiatory or injurious effect due to the excess. The optimum results usually followed treatment of 2 c.c. eggs in sea-water with 2 c.c. of one fourth or one eighth saturated iodine solution. While hypertonic after treatment was nearly always beneficial, in most cases the effect was so slight as to be negligible.

It may be recalled that membrane formation and subsequent

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cytolysis were obtained by Loeb,[1] McClendon,[2] and Glaser,[3] in *Arbacia* eggs, by placing them in sea-water diluted with distilled, or in pure distilled water. In the latter case, the eggs became "ghosts" of their former selves, since the pigment dissolved out and increased osmotic pressure caused the egg to absorb water and swell enormously. If an excess of iodine is added to normal eggs, they also become pale, but due to a precipitation of the pigment, which gathers as a small dark mass near one side of the egg. There is seldom indication of swelling or ghost formation.

The membranes obtained by the iodine method were identical with the true fertilization membranes which appear when the sperm enters the egg. They do not resemble the membranes formed by treatment with butyric acid and hypertonic sea-water, which Loeb describes as very difficult to see, excepting by the trained observer. This normal appearance of the membranes was noted by others accustomed to distinguishing types of membrane in *Arbacia*. The significance of the nature of this membrane will be discussed in a later paper.

Reference to Table I. brings out a result that was wholly unexpected. It is commonly supposed that eggs shed by the animal are in better condition and more fertile than those taken from the ovary. The results with one female, whose shed eggs produced only 3 per cent. cleavages, while those shaken from the same ovaries gave 94 per cent., were so striking that the averages were computed. It was found that the shed eggs averaged only 43 per cent. as against 80 per cent. cleavages from the eggs shaken from the ovaries. It is planned to test this during the height of the breeding season, so as to discover whether this is a normal occurrence or whether the "shed" eggs are more likely to be "over-ripe" than the others, late in the season.

[1] Loeb, Jacques, "Die chemische Entwicklungserregung, etc.," Julius Springer, Berlin, 1909.

[2] McClendon, J. F., "On Artificial Parthenogenesis of the Sea-urchin Egg," *Science*, 1909, Vol. 30, pp. 454–455.

[3] Glaser, O. C., "On Inducing Development in the Sea-urchin (*Arbacia punctulata*), Together with Considerations on the Initiatory Effect of Fertilization," *Science*, 1913, Vol. 38, pp. 446–450.

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BIOLOGICAL BULLETIN

SELECTION FOR HIGHER AND LOWER FACET NUMBERS IN THE BAR-EYED RACE OF DROSOPHILA AND THE APPEARANCE OF REVERSE MUTATIONS.[1]

H. G. MAY.

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THE EXPERIMENTS OF ZELENY AND MATTOON.

In 1915 Charles Zeleny and E. W. Mattoon published their results obtained by selecting for higher and lower facet numbers in the bar-eyed race of *Drosophila*. Three lines were selected for higher numbers and three lines for lower numbers. Individual pairs were mated and the selection was carried on for three generations in each direction.

They counted the facets in 250 flies at the beginning of the experiment and found the average number to be 98.04 for males and 65.06 for females. Counts made on 250 more flies from the stock at the end of the experiment gave the same results. From the ratio between numbers from males and females they computed a factor for converting the female number to the male number and published all numbers on the male standard. Without exception each generation produced successively higher

[1] Contribution from the Zoölogical Laboratory of the University of Illinois, No. 97.

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numbers in the upward selected lines and successively lower numbers in the downward selected lines, and in each case the three lines were very close together. The average facet number was raised from 98.0 to 139.5 and lowered from 98.0 to 83.7. The maximum was raised from 182 to 213 and the minimum was lowered from 45 to 36. This increase in the range was not due to a larger number of counts, as 500 flies were counted from the stock and only 450 from the selected lines.

The Experiments of MacDowell.

In the same year E. Carlton MacDowell published the results of selections for higher bristle numbers in a race of *Drosophila* with extra bristles on the thorax. For six generations the number of extra bristles increased, but failed to rise any higher in forty additional generations. The response to selection was not so definite as in the experiments of Zeleny and Mattoon. The variation in the averages from different lines was very large. While the average of a large number of lines increased with each generation, that of any particular line often decreased for a generation. This wide range of fluctuations in the averages from different lines was partly explained by the existence of a correlation between the number of extra bristles and the size of the fly.

There is, however, another possible cause that was apparently overlooked. MacDowell selected for extra bristles in two arbitrarily limited rows on the dorsal side of the thorax, but states that even when no extra bristles were present in those rows "these flies frequently showed extra bristles on other parts of the thorax." The factors for extra bristles evidently controlled not only the number of extra bristles in those rows, but also the number of extra bristles on other parts of the thorax and possibly over the entire body. Selection, then, was made for only a small part of a variable character. A high number of bristles in the area under observation would in general indicate a high number of bristles on other parts of the body, but might actually be accompanied by a low number of extras elsewhere. Low offspring from high parents could be accounted for in that way. The efficiency of the selection would also

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decrease as the part of the character under observation decreased in comparison with the part not under observation.

The Present Experiments.

Since the experiments of Zeleny and Mattoon gave such clear results, but were interrupted before they led to a final conclusion, it seemed desirable to repeat them on a larger scale and continue them for a greater number of generations to determine if a pure line could be established and to study the changes in the existing factor or factor complex.

Material and Methods.

In order to have a check on any possible contamination a stock was selected which had a second recessive character, the vestigial wing, in addition to the bar eye. This stock also had the advantage of having on the average a lower facet number than the long-winged stock. But sterility in the race and the indefinite character of the results made it necessary, after a few generations, to return to the long-winged stock. In both cases the eye color was that of the normal wild fly.

The vestigial-winged, bar-eyed stock was designated as VBa; the long-winged, bar-eyed stock as Ba. The downward and upward selected lines in VBa were distinguished by l and h, in Ba by d and u respectively. Individual lines were distinguished by numbers. Any given mating received the number of the generation to which it belonged and a serial number corresponding to the number of matings made from that line in the given generation. In that way a number like Bau4f2–5, although cumbersome, gives nearly the whole pedigree of the mating involved. Since the lines are now clearly distinguished without the stock designations, Ba and VBa, those designations will frequently be omitted in this paper.

The material for the present set of experiments was obtained from Professor Charles Zeleny on whose advice the work was undertaken. To him the author is also indebted for a keen interest in the work and for many helpful suggestions.

All stocks were kept in large bottles while eight dram vials were used for individual pairs. The vials were plugged with

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cotton and in case of the long-winged race contained pieces of filter paper to prevent the flies from adhering to the food.

Throughout these experiments bananas were used as food. Only specimens with perfect skin were selected. The pulp was never allowed to come in contact with the outside of the skin

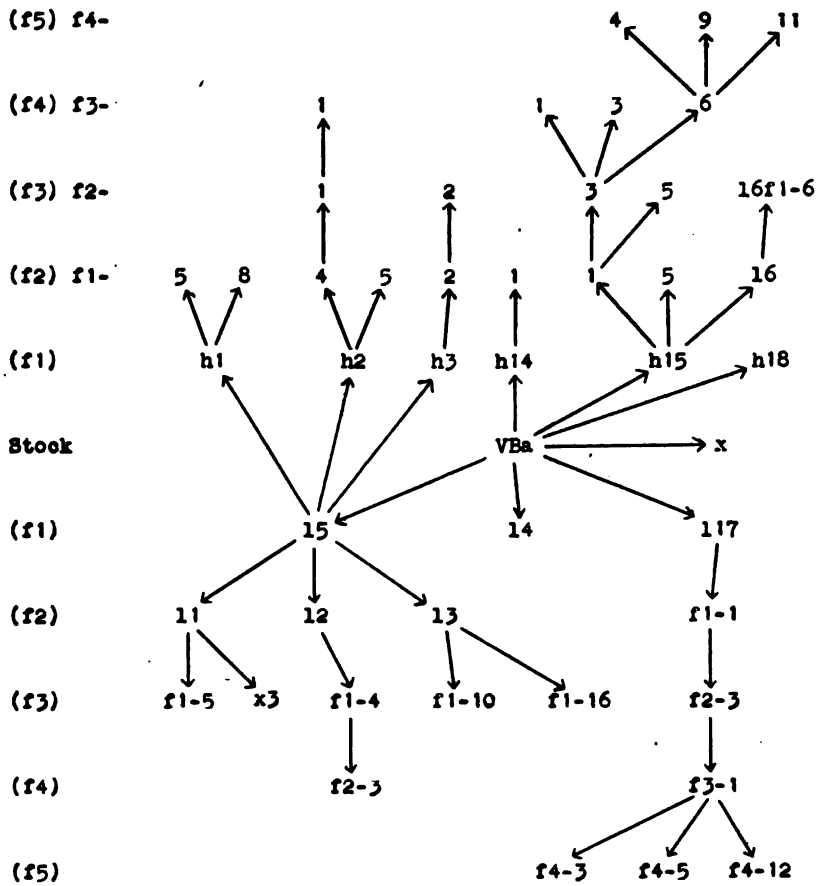

FIG. 1. Relation of selected lines and stock in VBa. In general the downward selected lines are below and the upward selected lines above.

and was heated to 70° C. and allowed to cool in a closed vessel before being placed into the food jar. Food was allowed to undergo alcoholic fermentation for about three days before being used. At first the author had considerable difficulty in keeping food, as acetic acid fermentation soon set in, the food

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became hard, and larvæ did not seem to thrive well in it. This defect, however, was remedied after one or two generations had been reared from the vestigeal-winged stock and long before the experiments on the long-winged stock were undertaken.

In some preliminary work the number of facets was estimated in the eyes of the parents selected and the actual counts were made at the time the parents were killed. This method was employed by Zeleny and Mattoon. The author, however, found that his estimates were not close enough and that in some cases parents were lost because they died and were destroyed by the larvæ before their death was discovered. The facets of dead flies also disintegrate when they remain in contact with food for a short time. For those reasons all selections during the experiments were made from actual counts.

During all of the work on the vestigial-winged flies and during part of the work on the long-winged flies, selections were made at noon and night after all the flies had been removed in the morning. In that way the specimens were never more than six hours old at the time of selection. But observations during that time showed that males seldom or never mated before they were twenty-four hours old, and for that reason later selections were made every twelve hours. All desirable specimens were saved and when no mates were present they were kept in vials with food until matings could be made.

All flies were etherized and the facets were counted while they were quiescent. The larvæ were never subjected to ether, as the flies to be examined were first transferred to empty vials. Selected flies recovered from the effect of the ether in fifteen minutes to half an hour. The other flies were preserved in 85 per cent alcohol.

For counting facets the flies were placed in a little pit on a paraffin block and illuminated by means of a 25-watt tungsten lamp. This gave sufficient light but only a moderate amount of heat. A Leitz microscope with a number four ocular and a number three objective and the tube drawn out to its full length was found to be most convenient. This gave a relatively high magnification with a suitable depth and size of field.

Errors in counting can not be avoided; it is merely a question

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of reducing them to a minimum. In eyes with 100 facets or less the error is certainly less than one per cent., but it increases rapidly as the facet number increases. Recounts made on eyes

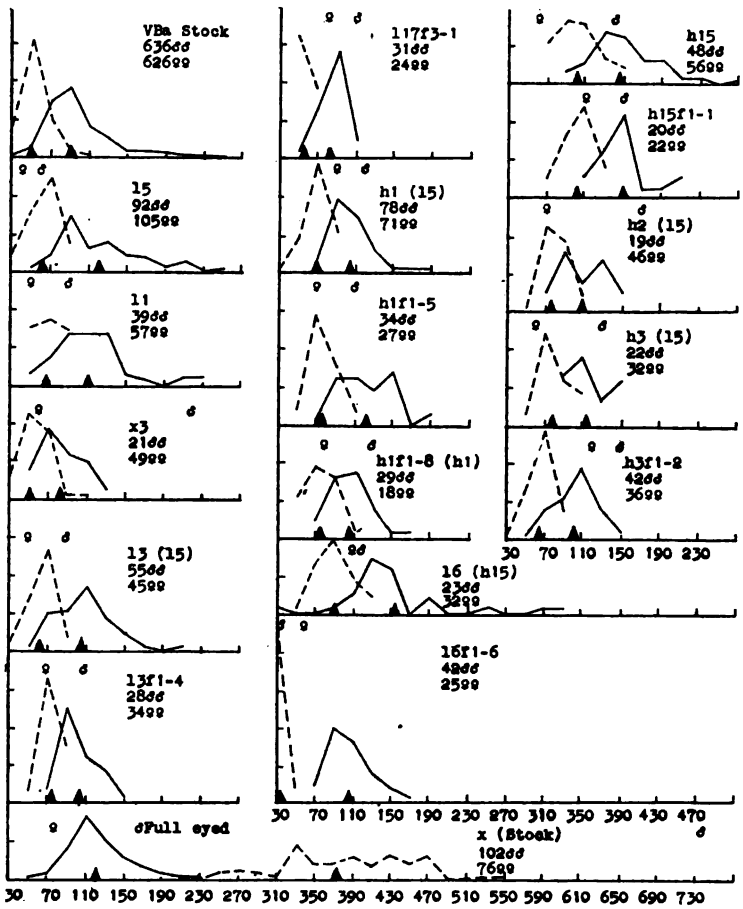

FIG. 2. Curves showing the effect of selection in individual lines in VBa. All curves are plotted on the scale of fifty. The males are represented by continuous lines and the females by interrupted lines. The parents are indicated at the top and the averages by the black pyramids below. The values plotted are for flies between 20 and 39, 40 and 59, 60 and 79, etc. The number of the mating is given above, followed by the source in brackets unless the parents come from the mating represented by the curve just above. In case of difficulty in determining the relationship of the lines consult Fig. 1.

with as many as 300 facets showed an error of possibly two or three per cent. Of course it takes two or three weeks of practice to reach that point of efficiency.

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Errors are due chiefly to the following causes: (1) The arrangement of the facets may be irregular. Ordinarily the facets are arranged in rows and one can easily count two rows at a time, even if the eye is large. But in the bar eye the dorsal part usually contains a large number of facets without any indication of rows. This area increases very rapidly with the size of the eye. In heterozygous females the facets are all arranged in beautiful rows, and one is thus able to distinguish readily between the eye of a heterozygous female and a bar-eyed male even if both eyes contain the same number of facets. Bar-eyed females as a rule contain more regular facets than the males and the distinction between low heterozygous females and high bar-eyed females is more difficult. (2) There may be small facets either among the others or at the margins. These facets are most abundant in irregular eyes and may be one of the causes of irregularity. All grades are found from mere prominences that can only be seen with the most advantageous light to normal-sized facets. As a rule, however, there are few if any doubtful facets. (3) There may be colorless facets at the margins. Normally the colored area of the eye extends somewhat beyond the facet area, but in some rare cases a few facets may extend beyond it and these may be overlooked. (4) When the eye is so large that the fly must be turned to count all of the facets some of the facets in the middle may be recounted or omitted. All heterozygous females and some high grade males had to be turned. The error in this case also is not very great, as the bar eye as well as the heterozygous eye is almost divided in the middle. (5) Errors may also arise through a lack of concentration or through the inability to make the eye retain its position. These are purely personal elements.

No mechanical device was used for marking off the counted area from the uncounted area on the eye of the fly. The author relied entirely on the ability of his eye to follow the rows or, in the absence of rows, to mark off certain areas and hold them until the eye was counted. A cross-hair in the ocular was tried, but was found to be unreliable. It could be used if the eye of the fly presented a flat field, but with the rounded contour of the eye the hair keeps on traveling over facets as one focuses up

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and down and comes to rest on the same facets only in the same focal plane. But it is impossible to count all facets without large changes in the focus. In shifting the fly after a given area has been counted it is evident that the same shift makes the hair pass over more facets on a slanting area than on a horizontal one, just as surveyors will pass over more surface in surveying up or down a hill than on the level. But on the rounded eye the hair usually passes over both a horizontal and a slanting area at the same time. For that reason one can not possibly shift so that the hair crosses the same number of facets at every point on the eye. A cross-ruled ocular has little or no advantage over a cross-hair, and a camera lucida is scarcely worthy of consideration as it is difficult enough to keep it at the proper place even on a perfectly flat field.

The largest error, by far, is due to the fact that only the right eye was counted. Zeleny and Mattoon reported that they found the averages of a large number of counts to be the same for both eyes. The author obtained the same results. But that does not mean that both eyes in any given fly have the same number. Normally the variation is not more than about 1 per cent from the mean, but the author has found it as high as 5 per cent, and in one case 10 per cent. One abnormal male was obtained with 25 facets in the right eye and 146 in the left. It is obvious that the error is greatest in the parents and the extremes of the offspring as we are dealing in those cases with individual flies. In parents the left eye was usually examined to see that it was not abnormal, and actual counts were made in a large number of cases. The abnormal male was mated to see if the unequal condition would be inherited, but it died within twenty-four hours without giving any offspring.

Selections in the Vestigial-Winged Stock.

When the bottle that was to give rise to the VBa stock was received, flies were transferred to two fresh stock bottles and the offspring from these parents were used for making the original selections. From October 31 to November 5 some preliminary counts were made on flies that hatched in the original bottle. Sixty-nine males gave a mean facet number of 115 and seventy females gave a mean of 63.

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All of the offspring from the parents placed into the new stock bottles were counted, including the ones that were used to continue the stock, and selections were made during the entire period. From the preliminary counts it was concluded that flies above a certain limit could be selected as high and those below another limit as low; but when the selections were begun it was found that no flies appeared that approached the lower limit set for high selections and many flies were below the limit set for low selections. As a result a new standard had to be established. Later the stock became more variable and some higher flies appeared together with some very low ones; indeed the lowest female obtained during these experiments was taken from one of these bottles. In all 370 males gave an average of 96 facets and 364 females gave an average of 54 facets. More counts on the stock were made March 17 to April 2, and this time 197 males gave an average of 82 facets and 192 females, an average of 44. This variability of the stock was contrary to the conditions found by Zeleny and Mattoon and seriously interfered with the success of the present experiments. It was necessary to pay as much attention to the nature and possible cause of the variation as to the effect of selection.

The greatest difficulty encountered in the VBa selections, however, was sterility and low production in the single lines. Of 42 matings made from the stock only 6 were fertile and of these only 2 gave a sufficient number of offspring for selection. On the whole about 80 per cent of the matings were sterile, and in many cases even lot matings failed to produce any offspring. By the end of the fifth generation all lines had died out except l17 and h15, and these were saved only through lots. For that reason the experiment was discontinued at that point.

No special experiments were made to determine the cause of sterility. Obviously it was not due to inbreeding, for it was as great in the first generation as in succeeding ones. In the majority of cases it was also not due to the inability of the females to deposit eggs as was the case in the experiments of Hyde. A few of such abnormal females were observed, but in most cases eggs appeared in the food and failed to hatch. The

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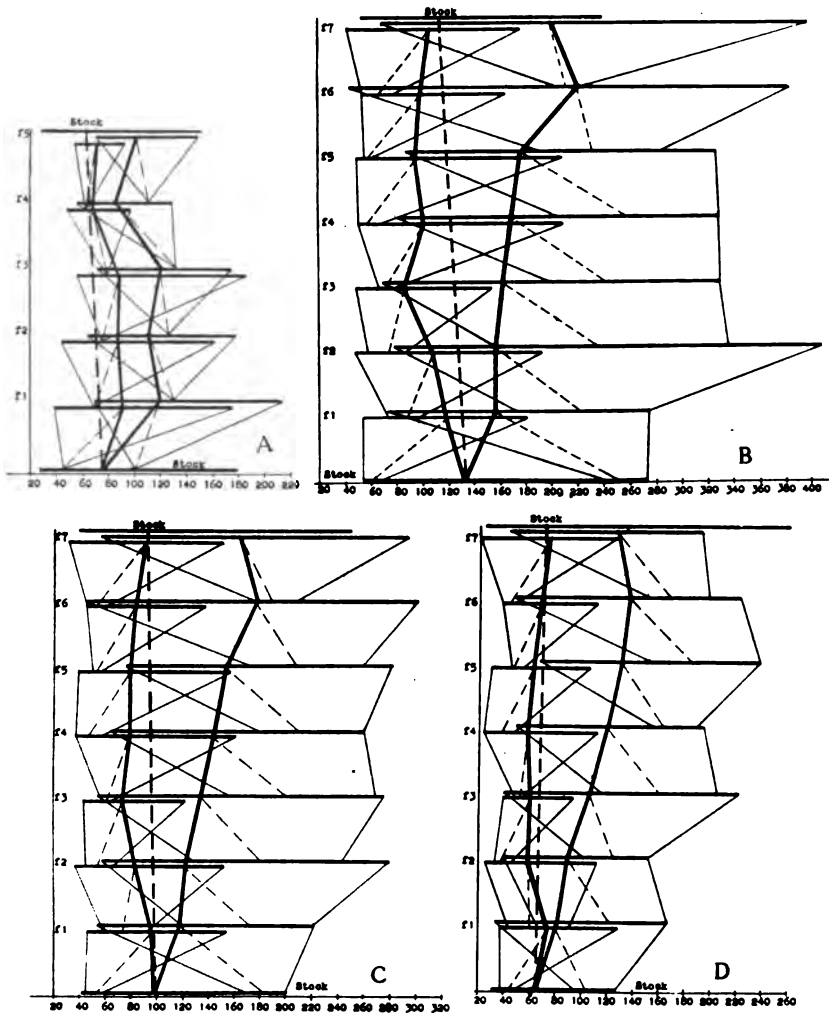

FIG. 3. The effect of selection on range and average. A, high and low lines in VBa; B, males in Ba, high and low lines; C, high and low lines in Ba; D, females in Ba, high and low lines. A and C are plotted for the means between male and female averages. These figures do not represent individual lines, but include all lines except reverse selections for the generations indicated. The heavy horizontal lines indicate the range, the heavy vertical lines, the averages. The heavy interrupted vertical lines merely connect the initial and final stock averages and do not indicate the averages of the stock at intermediate points. The fine slanting lines connect the extremes of the parents with those of the offspring while the fine interrupted lines connect the averages of the parents with those of the offspring. The facet numbers are indicated at the bottom, the generations at the left hand side.

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results of Castle correspond more nearly to the facts observed.

The results obtained from these selections can best be made out by consulting Table I. and Figs. 1, 2 and 3.

The attempt to select downward was entirely unsuccessful. The selection was never very rigid, but the average of the offspring persistently remained above that of the stock.

The upward selections were more successful. In h15 the males rose from the stock average of 96 to 147 and the females from 54 to 101. In the other two lines where smaller numbers of offspring were obtained the rise was not quite so large. The second and third generations, however, failed to give any further increase in the facet number. Upward selections taken from line l5 after the first generation failed to give any rise in the facet number. The line x3 was taken from l1 after two generations of downward selection and gave a decrease in the facet number in spite of the fact that the male had 222 facets and was mated to average females.

The line l6 was intended to be a downward selection from h15, but the parents proved to be very near the mean and all matings from the first generation were sterile except f1–6. This particular mating involved a male with 34 facets, the lowest number obtained in these experiments. Fortunately a fair number of offspring was obtained. The males average much lower than those of the previous generation and about the same as those of the low lines hatching at the same time, but the females average much lower than anything obtained elsewhere. It is likely that the male with 34 facets was a mutant and that the mutation chiefly concerned the sex chromosome. The complete sterility of this race prevented any further investigation.

The results here obtained are very different from those reported by Zeleny and Mattoon. In their selections all three generations went gradually upward and gradually downward; in these experiments there was a sudden rise in the upward selected lines in the first generation and no further effect, and no response at all in the downward selected lines. For that reason and also on account of the high degree of sterility it seemed best to make some selections in the long-winged stock used by Zeleny and Mattoon.

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Table I.

Relation of Parents and Offspring. Vestigial-Winged, Bar-Eyed Stock (VBa).

Parents.				Offspring.							
				♂				♀			
Bottle Number.	Source.	Facets.		No. Flies.	Facets.			No. Flies.	Facets.		
		♂	♀		Ave.	Max.	Min.		Ave.	Max.	Min.
Stock	Stock			636	93.8	253	36	626	51.9	108	19
l4	Stock	63	46	3	101.7	134	85	12	71.9	93	38
l5	Stock	60	45	92	120.2	254	56	105	63.2	98	23
l1	l5	86	48	39	110.3	222	57	46	67.4	95	41
l1f2	l1	64	46	10	116.5	269	77	12	73.8	104	56
l2	l5	56	37	13	107.3	142	70	14	69.2	88	52
l3	l5	98	53	55	106.3	208	56	45	63.6	88	32
l3f1–4	l3	108	71	28	102.9	149	76	34	74.7	97	51
l3f1–10	l3	96	53	9	116.1	142	82	10	77.6	96	56
l3f1–16	l3	75	56	1	121.0			1	86.0		
l3f2–3	l3f1–4	78	51	2	57.0	58	56	5	50.2	54	47
l17	Stock	46	19	2	98.0	108	88	2	85.5	90	81
l17f2	l17	108	90	4	118.8	122	116	8	92.4	102	83
l17f2–3	l17f2	116	83	12	100.9	165	70	17	60.1	89	42
l17f3–1	l17f2–3	113	81	31	85.0	117	57	24	56.3	76	41
l17f4–3	l17f3–1	77	54	9	84.2	96	70	3	64.0	74	51
l17f4–5	l17f3–1	lot	lot	16	82.4	108	60	11	57.2	77	48
l17f4–12	l17f3–1	lot	lot	2	90.0	100	80	6	59.8	65	53
h1	l5	120	86	78	106.2	182	71	92	70.1	99	29
h1f1–5	h1	108	74	34	122.0	192	78	27	77.7	101	58
h1f1–8	h1	128	75	29	107.3	160	75	18	73.6	103	52
h2	l5	170	73	19	110.7	163	68	46	79.4	105	53
h2f2	h2	156	90	5	146.2	175	125	6	89.3	113	79
h2f1–5	h2	163	100	4	88.0	112	72	6	58.7	69	48
h2f2–1	h2f2	150	113	2	103.5	115	92	6	59.7	70	50
h2f3–1	h2f2–1	115	70	14	109.6	143	88	13	67.4	89	55
h3	l5	126	64	22	114.8	154	82	32	80.0	116	57
h3f2	h3	145	116	42	102.7	150	52	36	67.4	95	29
h3f2–2	h3f2	122	90	3	80.3	85	72	1	65.0		
h14	Stock	155	64	9	128.3	190	95	3	89.0	108	77
h14f2	h14	161	108	4	94.0	113	74	9	69.0	82	63
h15	Stock	137	60	48	147.1	271	81	56	101.2	158	70
h15f1–5	h15	123	96	17	121.7	154	84	14	83.6	113	56
h15f2	h15	148	112	20	149.7	219	105	22	102.5	137	64
h15f2–3	h15f2	lot	lot	16	147.2	199	92	19	91.4	125	51
h15f2–5	h15f2	205	111	16	151.6	217	118	8	94.0	129	57
h15f3–1	h15f2–3	156	109	10	96.7	126	76	8	73.5	123	59

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Parents.				Offspring.							
				♂				♀			
Bottle Number.	Source.	Facets.		No. Flies.	Facets.			No. Flies.	Facets.		
		♂	♀		Ave.	Max.	Min.		Ave.	Max.	Min.
h15f3-3	h15f2-3	150	107	3	84.0	97	62	3	76.7	85	61
h15f3-6	h15f2-3	158	102	9	97.0	134	63	19	73.8	103	50
h15f4-4	h15f3-6	131	103	7	120.6	196	79	4	89.3	100	65
h15f4-9	h15f3-6	lot	lot	3	117.3	144	88	3	78.7	87	63
h15f4-11	h15f3-6	134	93	2	124.0	131	117	3	74.3	86	63
h18	Stock	163	62	12	123.8	241	90	11	94.8	129	59
16	h15	122	108	23	151.3	338	34	32	89.6	127	56
16f1-6	16	34	64	42	105.1	170	70	25	33.5	56	25
x	Stock	full	43	102	122.3	228	56	76	377.9	543	218
x3	l1	222	lot	21	82.0	127	56	49	54.9	104	31
x4	16	328	lot	12	142.0	243	70	16	77.4	116	53
x4f1-1	x4	140	116	5	146.0	181	130	10	111.5	135	83
x4f2-5	x4f1-1	132	103	6	169.5	196	143	1	119.0		

Selections in the Long-Winged Stock.

These experiments were begun on January 15 and continued until the middle of June. In this case the number of flies counted from the stock during selection was 91 males with an average facet number of 132 and 81 females with an average of 66. The first selection made was very near the mean and was designated as Ba1 and used as part of the stock. It yielded 44 males and 37 females with mean facet numbers of 125 and 68 respectively. In June the eyes of 73 additional males and 97 additional females from the stock were counted, yielding mean facet numbers of 114 and 72 respectively. Here again, as in the vestigial-winged stock, there is a change in the mean facet number, and in this case it also involves a decided change in the ratio between male and female facet numbers.

Little difficulty was encountered on account of sterility in this stock. About 75 per cent of the matings were fertile and nearly 75 per cent of the fertile matings produced 50 offspring or more. In each case all of the offspring were counted. To avoid the dying out of lines a number of branches of each line was run, only brothers and sisters were mated, but no attention was paid to the dying out of any given branch when it did not

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come up to the standard in facet number or production. In that way the most suitable flies from the most suitable branches were selected, bringing the selections to some extent on the behavior basis. By that method parents whose somatic constitution varies from their germinal constitution are to some extent eliminated.

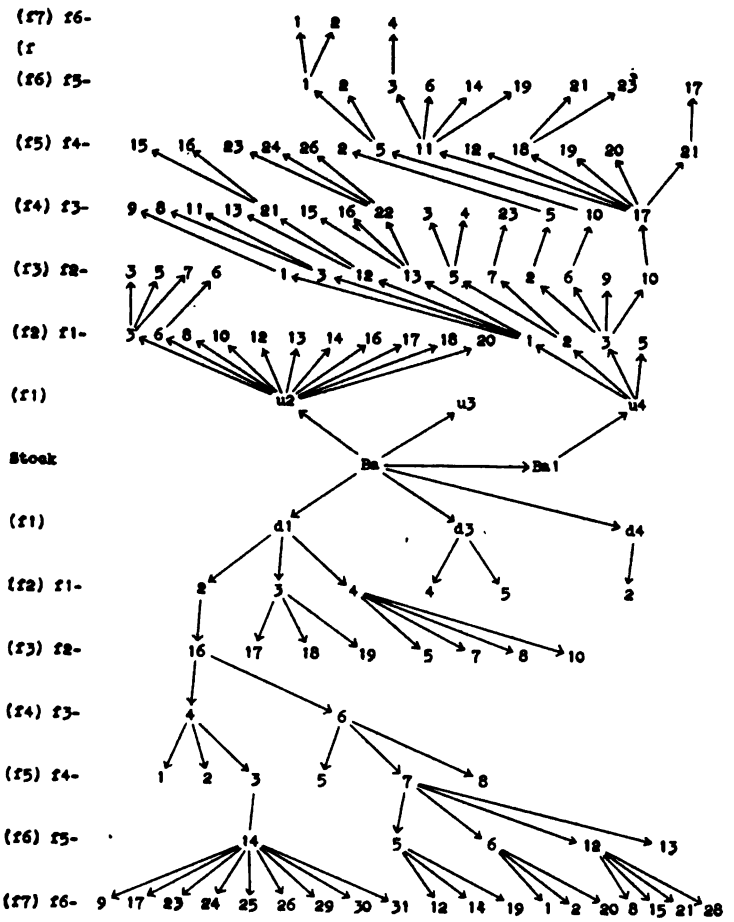

FIG. 4. Relation of selected lines and stock in Ba.

Selections in this stock were carried on for seven generations in each direction, several return selections were made, and crosses in both directions were finally made from the selected lines. The results are shown in Table II. and in Figs. 3, 4, 5, 6 and 7.

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The downward selections were not very effective. In the first generation the average facet number in the females actually rose above that of the stock, but the average of the males dropped

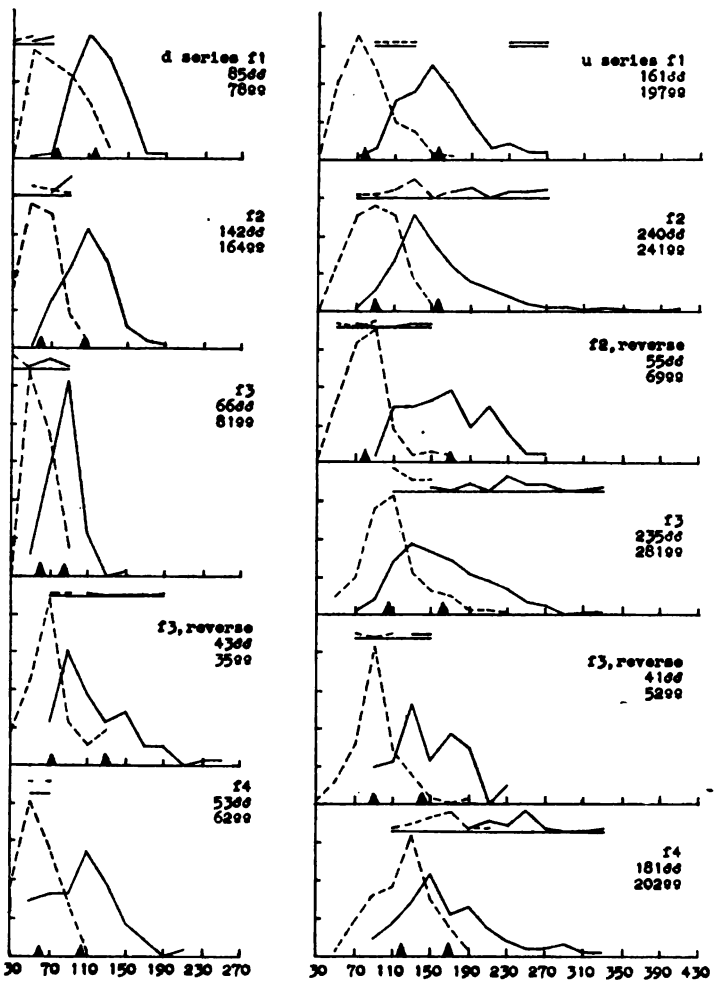

FIG. 5A.

considerably below. In the next generation the females dropped decidedly and the males dropped only slightly. The third generation produced another slight lowering of the means, and after that there was a gradual return toward the mean of the stock.

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In the seventh generation the mean of the stock had been reached by the females, but the males were still slightly lower. Taking the mean of the male and female averages more uniform results appear to have been obtained. In that case the first generation shows no change, the second and third generations show a gradual

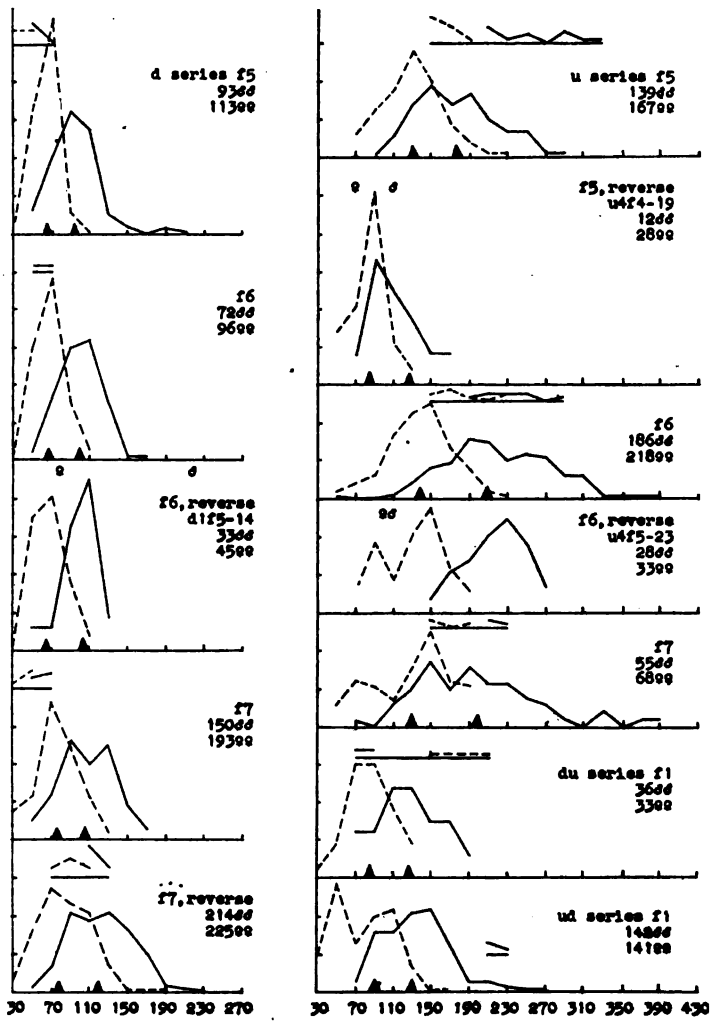

FIG. 5*B*.

FIG. 5. Curves showing the effect of selection for generations in Ba. These curves are plotted on a scale of one hundred. Parents are indicated by smaller curves at the top. In other respects the curves are like those of Fig. 2.

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lowering, but the next four generations show a complete return to the mean of the stock.

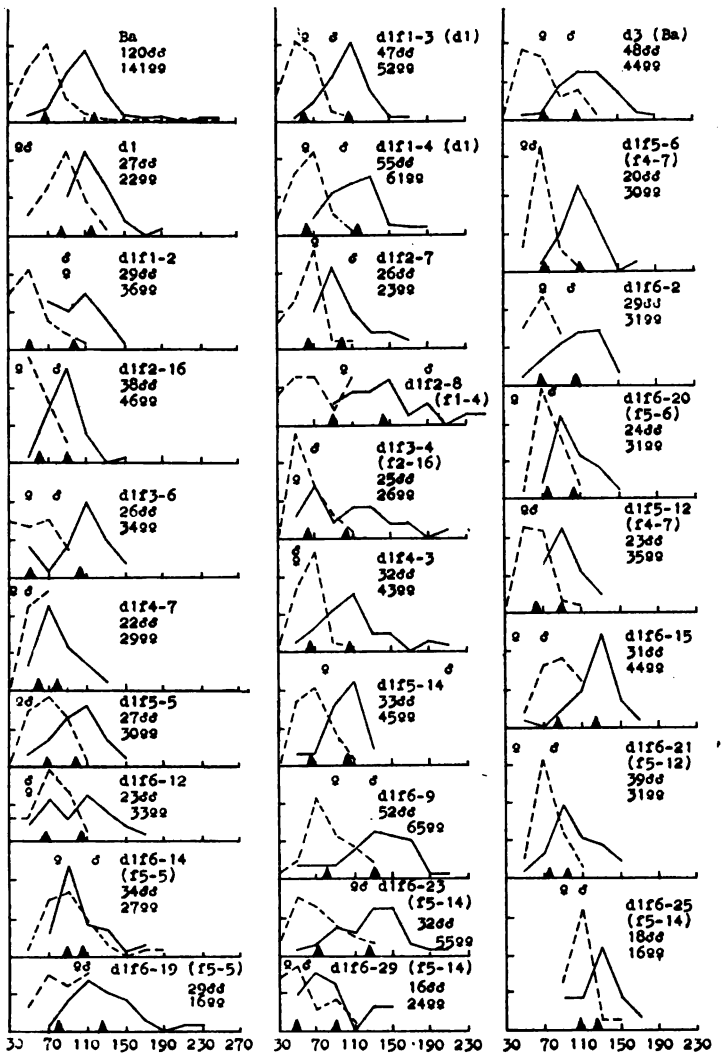

FIG. 6. Curves showing the effect of selection in individual lines in Ba, downward selections. For other information consult Fig. 2.

The upward selections were more successful. With the exception of the last generation there is in every case a slight rise

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in both sexes. The results in the last generation can be explained by the fact that the selections were below the average

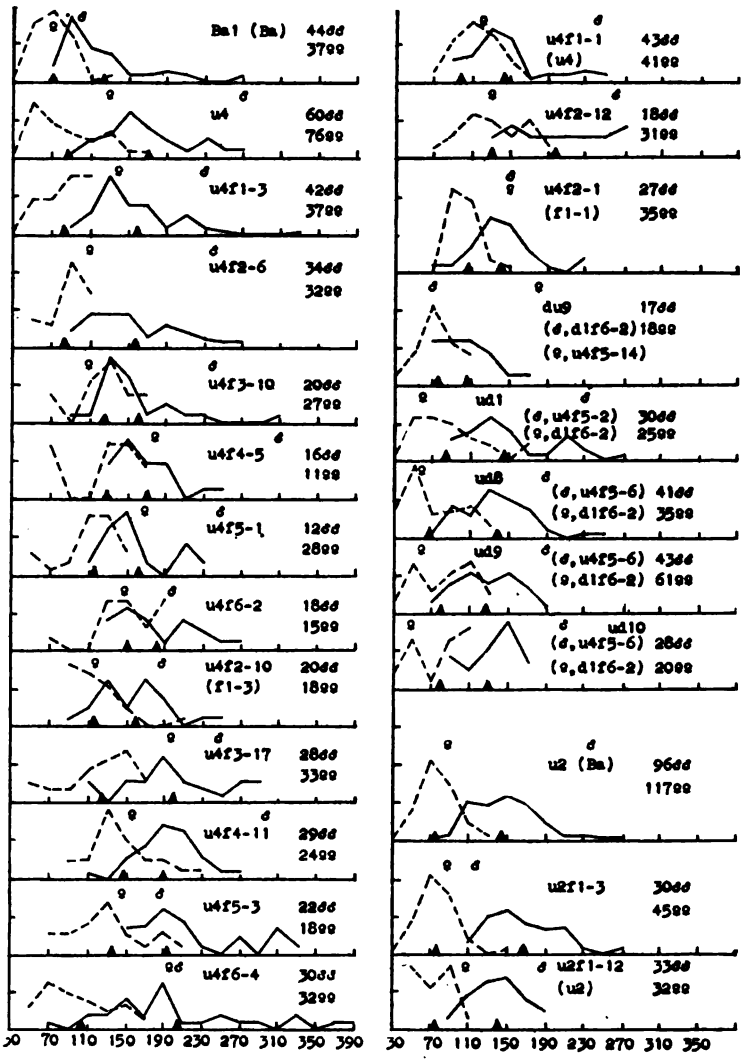

FIG. 7. Curves showing the effect of selection in individual lines in Ba, upward selections and crosses. For other information consult the description of Fig. 2.

in the males and not far above in the females, and the fact that the offspring hatched at a time when all counts were low. This will be explained later.

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TABLE II.

RELATION OF PARENTS AND OFFSPRING. LONG-WINGED, BAR-EYED STOCK (Ba).

Parents.				Offspring.							
				♂				♀			
Bottle Number.	Source.	Facets.		No. Flies.	Facets.			No. Flies.	Facets.		
		♂	♀		Ave.	Max.	Min.		Ave.	Max.	Min.
Ba	Stock			120	122.7	240	53	141	69.1	259	24
Ba1	Stock	104	74	44	125.0	273	69	37	68.3	128	34
d1	Stock	53	38	27	117.4	180	91	22	84.0	122	56
d1f1–2	d1	92	91	29	99.5	141	64	36	52.5	102	25
3	d1	92	56	47	106.0	164	58	52	58.4	108	28
4	d1	98	65	55	114.7	192	62	61	59.4	111	32
d1f2–5	d1f1–4	84	62	0				1	65.0		
7	4	106	73	26	97.9	162	62	23	62.6	117	37
8	4	190	98	17	148.6	242	85	12	90.0	131	47
10	4	87	47	11	87.1	104	67	11	63.4	87	48
16	2	76	36	38	87.6	153	58	46	61.6	92	40
17	3	75	36	12	72.3	95	49	17	54.5	71	38
18	3	68	35	4	80.0	87	73	6	63.7	73	58
19	3	58	33	1	109.0			1	64.0		
d1f3–1	d1f2–10	76	48	2	88.5	90	87	2	50.0	53	47
4	16	68	54	25	103.2	209	51	26	60.6	111	26
6	16	79	53	26	101.8	154	57	34	55.1	98	23
d1f4–1	d1f3–4	57	48	11	98.9	122	74	7	64.6	68	59
2	4	51	44	11	90.2	107	79	13	70.2	88	49
3	4	55	47	32	109.4	208	55	43	62.2	104	37
5	6	59	32	4	87.0	96	75	6	66.8	82	37
7	6	57	23	22	80.3	133	56	29	59.1	77	29
8	6	69	35	13	91.2	115	50	15	63.5	91	49
d1f5–5	d1f4–7	56	45	27	98.9	155	54	30	70.8	100	37
6	7	61	46	20	111.5	165	78	30	72.3	105	45
12	7	56	55	23	90.2	127	65	35	61.3	110	39
13	7	62	51	2	78.5	83	74	1	71.0		
14	3	208	77	33	101.1	132	56	45	66.6	116	30
d1f6–1	d1f5–6	165	105					1	67.0		
2	6	102	70	29	108.2	155	59	31	69.7	99	42
8	12	126	110	2	102.5	103	102	3	85.0	90	81
9	14	125	93	52	131.5	215	50	65	82.0	139	27
12	5	54	49	23	103.7	166	42	33	69.3	104	22
14	5	122	83	34	103.3	164	69	27	93.8	187	52
15	12	68	45	31	123.4	167	51	44	86.1	114	44
17	14	119	92	1	121.0						
19	5	111	100	29	127.3	237	71	16	84.6	117	52
20	6	78	45	24	101.9	144	72	31	78.9	112	49
21	12	78	39	39	97.4	153	51	31	72.7	104	40
23	14	116	110	32	125.3	206	51	55	70.9	135	23

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TABLE II.—*Continued.*

Parents.				Offspring.							
				♂				♀			
Bottle Number.	Source.	Facets. ♂	Facets. ♀	No. Flies.	Facets. Ave.	Facets. Max.	Facets. Min.	No. Flies.	Facets. Ave.	Facets. Max.	Facets. Min.
d1f6–24	14	114	94	13	135.5	185	81	16	104.2	127	73
25	14	113	90	18	125.1	163	89	16	107.5	145	86
26	14	108	66	20	105.0	147	51	7	87.3	121	49
28	12	77	48	14	127.1	176	72	24	88.0	125	30
29	14	57	43	16	90.8	158	56	24	53.9	109	20
30	14	117	72	13	115.5	178	78	19	65.8	105	42
31	14	56	30	3	88.3	95	83	6	71.2	90	46
d3	Stock	65	51	48	118.2	181	54	44	72.8	128	37
d3f1–4	d3	74	43	1	92.0			7	72.0	92	54
5	d3	84	45	2	59.0	70	48	1	45.0		
d4	Ba1	69	48	10	116.3	145	81	12	66.9	86	50
d4f1–2	d4	90	61	8	128.6	157	108	5	64.2	78	54
u2	Stock	238	94	96	147.6	268	74	117	77.5	132	39
u2f1–3	u2	122	89	30	165.7	261	103	45	75.2	141	32
6	u2	144	94	15	187.7	249	107	14	90.9	162	52
8	u2	172	92	28	172.1	408	79	26	90.2	148	59
10	u2	187	132	22	191.8	330	125	14	104.1	144	69
12	u2	190	111	33	141.6	191	86	32	74.7	113	43
13	u2	192	109	16	144.6	203	90	18	94.4	135	49
14	u2	220	110	2	175.5	186	165	4	116.5	130	105
16	u2	246	123	8	126.3	153	106	10	95.1	111	75
17	u2	268	126	5	206.6	278	130	4	116.3	124	110
18	u2	172	63	22	141.7	249	78	38	81.0	138	49
20	u2	74	56	10	127.1	221	90	10	90.5	100	77
u2f2–3	u2f1–3	190	102	15	157.0	240	88	16	91.3	116	72
5	3	261	146	1	136.0			2	121.5	134	109
6	6	220	114	4	114.0	120	104	2	81.0	88	74
7	3	136	76	6	107.7	138	81	6	77.0	88	68
u3	Stock	240	109	5	124.8	138	98	4	101.8	120	93
u4	Ba1	273	128	60	170.9	275	99	76	85.3	167	34
u4f1–1	u4	241	121	43	146.3	255	83	41	94.5	151	49
2	u4	271	160	18	154.2	274	100	15	105.5	140	66
3	u4	227	136	42	158.2	336	96	37	83.1	116	39
5	u4	275	167	1	161.0			2	100.5	118	83
u4f2–1	u4f1–1	153	151	27	141.4	231	74	35	106.0	173	61
2	3	147	106	35	153.3	221	93	46	92.1	191	35
3	1	225	116	51	151.6	331	70	41	87.3	144	40
5	2	223	140	13	179.4	224	112	11	110.0	173	51

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TABLE II.—*Continued.*

Parents.				Offspring.							
				♂				♀			
Bottle Number.	Source.	Facets.		No. Flies.	Facets.			No. Flies.	Facets.		
		♂	♀		Ave.	Max.	Min.		Ave.	Max.	Min.
u4f2–6	3	237	111	34	156.9	263	83	32	86.4	113	40
7	2	274	129	31	166.1	238	107	54	109.9	217	45
9	3	336	100	8	179.1	229	150	11	108.3	169	85
10	3	187	118	20	156.8	244	99	18	114.8	205	81
12	1	255	127	18	199.1	266	126	31	131.0	190	66
13	1	252	122	13	203.4	267	119	28	126.3	221	69
u4f3–3	u4f2–5	215	173	3	135.7	165	99	6	147.0	193	84
4	5	213	147	7	160.4	182	125	14	124.9	166	61
5	2	197	121	30	173.1	330	92	23	112.1	154	75
8	3	331	173	7	142.9	198	108	2	151.5	166	137
9	1	231	143	3	148.0	190	117	6	124.7	158	113
10	6	241	112	20	159.2	308	99	27	126.5	176	64
11	3	242	136	8	156.5	207	129	7	115.3	127	96
13	12	260	190	12	194.7	291	140	14	126.2	150	89
15	13	245	172	4	149.5	185	113	14	114.6	148	83
16	13	221	172	9	139.3	167	112	6	102.0	136	70
17	10	244	205	28	201.0	298	108	33	122.2	168	48
21	12	255	179	18	175.5	294	132	16	122.0	167	65
22	13	255	145	15	178.9	244	99	25	115.6	190	67
23	7	216	145	17	127.1	281	82	9	94.3	149	75
u4f4–2	u4f3–5	330	154	22	159.3	260	88	20	99.7	141	67
5	10	308	176	16	169.9	240	129	11	125.6	173	69
11	17	290	157	29	191.6	269	101	24	145.3	236	81
12	17	298	158	5	144.4	168	120	4	129.5	141	125
15	21	203	156	2	165.5	167	164	7	114.0	137	77
16	21	220	165	4	155.0	187	138	4	138.5	158	129
18	17	284	156	15	190.6	284	106	27	134.2	237	77
19	17	109	67	12	128.9	192	77	28	84.1	127	52
20	17	250	155	3	153.7	196	109	6	120.2	141	86
21	17	209	152	11	186.0	256	132	8	141.1	195	84
23	22	214	163	13	173.7	257	125	17	123.2	160	78
24	22	244	190	17	186.8	327	130	37	136.1	182	85
26	22	209	161	2	120.0	121	119	2	131.5	135	128
u4f5–1	u4f4–5	238	173	12	158.3	222	111	28	113.3	153	46
2	5	240	167	14	182.2	235	118	10	135.3	162	76
3	11	189	153	22	195.6	302	123	18	133.9	209	76
6	11	213	151	42	225.2	384	45	37	127.6	177	65
14	11	249	182	43	247.0	367	143	50	137.7	198	72
17	21	205	162	31	242.2	354	151	28	148.5	201	58
19	11	231	236	14	215.7	303	140	40	151.0	221	92
21	18	284	237	8	192.5	210	170	7	151.3	185	103
23	18	106	98	28	217.2	278	151	33	130.4	186	64
u4f6–1	u4f5–1	222	153	7	213.3	260	116	21	152.2	191	81
2	1	201	149	18	184.1	262	128	15	149.3	191	76
4	3	209	196	30	204.4	398	69	32	99.1	174	43

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TABLE II.—*Concluded.*

Parents.					Offspring.							
			Facets.		♂				♀			
Bottle Number.	Source.		♂	♀	No. Flies.	Facets. Ave.	Max.	Min.	No. Flies.	Facets. Ave.	Max.	Min.
du1	6–2	4–11	67	200	12	148.3	198	116	9	98.0	122	67
2	5–12	5–2	82	151	6	146.8	178	110	5	95.4	124	60
8	6–2	5–3	88	171	1	135.0			1	112.0		
9	6–2	5–14	74	182	17	104.6	160	66	18	73.5	113	39
ud1	5–2	6–2	235	59	30	149.1	264	81	25	83.8	166	32
8	5–6	6–2	205	56	41	138.2	245	61	35	69.1	123	31
9	5–6	6–2	205	56	43	127.1	187	72	61	81.3	129	30
10	5–6	6–2	211	54	28	132.0	169	85	20	80.1	113	28

TABLE III.

DAILY COUNTS, CROSSES BETWEEN LOW MALES AND HIGH FEMALES.
(Males from d1, Females from u4.)

Date.	Badu1.				Badu2.				Badu8.		Badu9.			
	♂		♀		♂		♀		♂	♀	♂		♀	
	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.			Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.
My 24					117		124 110							
25	198 133 147				149 110 166									
26	163		122		178					112				
27	157 186	134	121 99		161				135		136 113		100 84 89	
Je 6	131 117 158 139 116		76 67 114 97	89 97				60 97 86			148 134 110 160 99 111 102 95 123 93	67 82 68 71 66	39 113 86 69 60 92 63 54 61 52 69 103 70 70	49

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TABLE IV.

DAILY COUNTS, CROSSES BETWEEN HIGH MALES AND LOW FEMALES.
(Males from u4, Females from d1.)

Date.	Baud1.				Baud8.				Baud9.				Baud10.	
	♂		♀		♂		♀		♂		♀		♂	♀
	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.	Bot. 1.	Bot. 2.		
My 24	159													
	148													
25	200		133		144		121		158		104			
	238		161		138		112		121		126			
	264						116				98			
	224										115			
											113			
26	164		166		163		119		149		116		152	93
	211		118		125		123		179		96		169	102
	203		123		133		104		187		120		168	96
					153				175		113		120	
					143				174		98		127	
					136				144		119		157	
					136				116		105		133	
											109			
											127			
27	133		106		177		89		172		111		161	93
	184		105		169				169		122		161	113
	136		71		155				183		98		144	106
	132		82		245				178		116		131	113
	205		80		138				153		112		146	112
	129				198				153		111		155	112
					142						113		149	113
					153						80		146	
											108			
											129			
											124			
Je 7, 8	104	147	84	58	166	92	49	34	102	97	66	43	94	28
	81	114	54	64	239	123	57	80	150	72	33	75	115	49
	126	133	83	99	158	107	57	76	140	116	54	85	140	88
	94	108	65	32	111	96	51	62	128	85	32	81	96	42
	154	141	72	40	151	170	77	45	91	135	53	51	139	83
	97	118	54	52	163	108	46	77	109	118	65	30	140	37
	136	101		56	96	98	47	54	72	76	41	47	128	70
		89		63	83	125	88	46	81	113	35	48	109	52
				73		114	87	52	127	121	60	81	89	44
						Full		41	92	103	66	81	104	56
						134		34	121	105	68	51	87	
						126		31	150	83	55	40	85	
						61		48		111	109	55	150	
						94		104		141	85	99		
						95		87		92	37	71		
						114		37		123	80	91		
						195		32			48	58		
								36			57	43		
								100						

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Return selections from the low lines produced on the average slightly higher numbers than low selections in the same generations. Individual cases, however, do not seem to show any definite response. The few return selections made from the

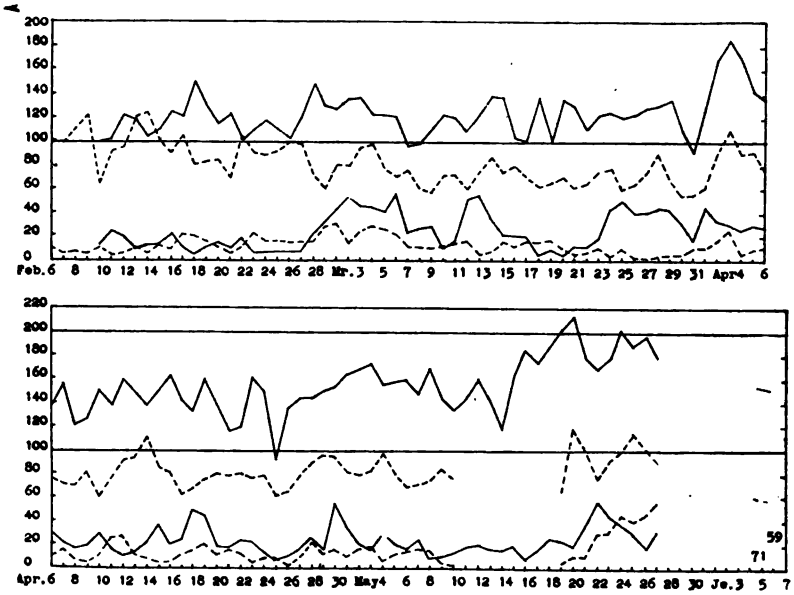

FIG. 8. Daily averages of high and low lines in Ba from February 6 to June 7, 1916. The high lines are represented by continuous lines and the low ones by broken lines. The two upper lines show the daily facet averages while the lower ones represent the actual numbers of flies from which those averages were obtained.

high lines were even less definite. Only one gave offspring that could be distinguished from those of the generation in which they hatched (u4f4–19).

The offspring from the crosses between low and high lines are intermediate and show a very slightly wider range than the low lines, but not nearly so wide a range as the high lines. There is no significant difference between the offspring from high males and low females and those from low males and high females. The apparent difference in the extremes is accounted for by the fact that the standard of all the lines changed during the period of hatching. The results of these matings are given in actual daily counts in Tables III. and IV., and are also shown in Figs. 5 and 7. A comparison of Tables III. and IV. with Fig. 8 shows

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that the lower values in the later counts are entirely in agreement with the results obtained at this time in the high and low lines.

Such changes in facet numbers of all the lines had already been noticed in the vestigial-winged race and are clearly shown at several points in Fig. 8. The changes would appear more sudden and the agreement between the lines would be closer if it were not for the fact that the change appeared sooner in bottles that just began to hatch than in older bottles.

The Appearance of Heterozygous Females and Full-Eyed Males.

What developed to be the most puzzling phase of the entire set of experiments was the appearance of full-eyed males and heterozygous females in the stock bottles and in the selected lines during the period they were under observation. Eleven such individuals appeared at different times as shown in Table V.

Table V.

Pedigree of Full-Eyed Males and Heterozygous Females Obtained from Bar-Eyed Stock under Observation.

Sex.	Date of Appearance.	Line.	Facets.	Fate.	Grade of Mates.	Offspring.				
						Bottle No.	♂		♀	
							Bar.	Full.	Bar.	Het.
♂	Nov. 15, 1915	VBa (Stock)	700?	Mated	43	VBax	102			76
♂	Nov. 29, 1915	VBa (Stock)	1200?	Died						
♂	Dec. 17, 1915	VBah15	1200?	Sterile						
♀	Jan. 16, 1916	VBah3f2	371	Mated	109, 116	VBah3f2–6	4	4	1	4
♂	Feb. 12, 1916	Bau2	1200?	Mated	61, 67	Bau2x	13			10
♀	Mar. 10, 1916	Bau2f2–3	601	Died						
♂	Mar. 28, 1916	Bau4f2–10	700?	Mated	149	Bau4f3x	27	17[1]	20[1]	18
♀	Apr. 2, 1916	Bau4f2–13	481	Mated	238, 204	Bau4f2–13x	3	3	2	4
♀	Apr. 28, 1916	Bau4f4–16	621	Mated	187	Bau4f5x	11	13	7	11
♀	June 6, 1916	Bau4f6–4	452	Killed						
♂	June 7, 1916	Baud8	800?	Killed						

[1] The full-eyed males and bar-eyed females in Bau4f3x were undoubtedly due to the appearance of flies from the second generation before the counts were made. The parents were mated April 3 and by an oversight the offspring were permitted to remain in the bottle until April 27.

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The VBa stock in March revealed no such specimens, but the Ba stock in June contained several of each. In that case they were probably due to the appearance of a heterozygous female in the previous generation. One of the stock bottles received from Professor Zeleny contained a mixture of bar-eyed, full-eyed and heterozygous individuals. These may have been due to the appearance of a full-eyed male or a heterozygous female at some previous time.

The eyes of the males could not be distinguished from those of normal wild flies. The facet numbers indicated in the table are mere estimates obtained by counting a row of facets to the middle of the eye and using the number obtained as a radius for computing the number on the entire area. The eyes of wild flies treated in the same way gave similar results. The eye of a wild male which could be counted without turning was found to contain 700 facets.

The eyes of the heterozygous females had the characteristic appearance of the eyes of such females obtained from crosses between wild and bar-eyed flies and contained the corresponding facet numbers. The ordinary bar eye has the shape of a crescent with a notch near the middle of the concave side almost separating the two ends of the crescent. One end is usually considerably larger than the other and frequently contains the irregular facets mentioned before. The eye of the heterozygous female has the shape of the bar eye, but is much larger and does not contain the irregular facets. As shown in the table, the heterozygous females from the long-winged race had considerably larger eyes than those from the vestigial-winged race. This is explained by the fact that the average facet number in the bar eye of the long-winged race is much larger than that of the vestigial-winged race.

Wherever possible the full-eyed males and heterozygous females that appeared were mated, but, with the exception of the first case, no attempt was made to get a large number of offspring or to count the facets in the bar-eyed and heterozygous offspring. Two of the individuals died soon after hatching and the last two could not be mated because the experiments had to be brought to a close. Of those mated one proved to be

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sterile and the other six produced offspring. In each case the specimens were mated with bar-eyed flies and with one exception the full-eyed males produced nothing but bar-eyed males and heterozygous females and the heterozygous females produced bar-eyed and full-eyed males and bar-eyed and heterozygous females. The one exception occurred in case of the male which appeared on March 28 and was mated to a bar-eyed female on April 3. The bottle was set aside and overlooked until April 27 when it was found to contain all four classes of flies as shown in the table. In that case the full-eyed males and bar-eyed females are undoubtedly due to the appearance of flies of the second generation before the count was made. Since the first generation consisted of bar-eyed males and heterozygous females the second generation would be expected to contain a mixture of all four classes.

The facets in the eyes of all the offspring obtained from the male appearing on November 15 were counted and the results are given under VBax in Table I. and Fig. 2. A large number of pairs from these offspring was mated, but all proved to be sterile. A mass mating made toward the end of the hatching period, however, produced 14 bar-eyed males with an average facet number of 114, 7 full-eyed males, 12 bar-eyed females with an average facet number of 91 and 5 heterozygous females. These were bred in a stock bottle for several generations to see if one or the other class would become dominant, but no obvious change in the ratios appeared.

Discussion and Conclusions.

The variability of the stock at the beginning of the selections made it appear probable that the facet number is affected by environmental conditions. For that reason the records during the experiments were kept more carefully than would otherwise have been the case. Counts were made and recorded separately at least every twelve hours. Each line or branch of a line was recorded separately, and in cases where parents were transferred and offspring obtained from both bottles a separate record was kept of each. Observations were also made on a possible correlation between body size and facet number.

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It was impossible to find any noticeable effect of moisture, consistency, or age of food on the facet number. No significant difference was found in facet number between the first flies hatching from a bottle and the last ones except in cases where the food gradually dried up and the last flies were minute, *i. e.*, one half the size of normal flies or less. Such minute flies showed a tendency toward lower facet numbers. Accurate size measurements and facet counts on a large number of flies may possibly reveal a slight correlation between size of body and number of facets, but mere observation failed to detect it. It is also possible that a slight correlation exists but is concealed by other factors that have more influence.

No relation could be discovered between the age of the parent and the facet number of the offspring. In case of such a relation the latest offspring from two parents would be different from the first. No such difference could be detected.

Likewise it was impossible to find any definite correlation between the length of the developmental period of the larva and the facet number of the adult. No special experiments were undertaken with material in which the exact developmental period was ascertained, but, since in productive bottles the period of hatching is much longer than the period during which the eggs are laid, the first larvæ must as a rule have a shorter developmental period than the last, and any difference due to the length of the developmental period should become evident.

A glance at Fig. 8 shows that beyond the daily fluctuations due to the fact that the number of flies examined was too small to be representative, there are larger, parallel fluctuations of the two lines. Since the food during the Ba selections was fairly constant in character it can not be regarded as the cause of these fluctuations. The temperature of the room in which the flies were kept varied between rather wide limits. Unfortunately no thermograph was available for the room at that time. There is no correlation between the outside temperature and the facet number; but the variations in the room temperature were independent of those in the outside temperature. Since experiments to determine the effect of temperature on the facet number were undertaken by E. W. Seyster of this laboratory during the

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latter part of this work and on account of the lack of time no special effort was made to determine the exact relation between temperature and facet number. The fact that the change usually occurred one to three days sooner in bottles that just began to hatch than in old ones can be explained by the assumption that the effect was produced at an early stage in the developmental period. Since the first flies hatching from a bottle must have a shorter developmental period than the later ones they would be the first to show the effect.

It does not seem possible, however, that temperature is the sole cause of somatic variations. In the VBa selections it was observed that the two lines throughout averaged higher than the stock. In spite of that fact an examination of the extremes shows that most of the high flies were eliminated from the low lines. The rank of the average flies must, then, have been raised. The same can be said of the low lines in Ba. Here the elimination of high flies is much more pronounced and still the mean remains very near that of the stock. It is possible that the crowded condition of the larvæ in the stock bottles reduced the facet number, but the real cause may be something very different.

The results of the selections in VBa indicate that there is in this race only a single hereditary factor involved in the modification of the facet number. In spite of the fact that the mean of the stock is much lower than that of either line we must assume that under the same conditions it would lie somewhere between them, in other words, if the lines could have been reared under the conditions of the stock, the low lines would have gone slightly downward due to the elimination of high numbers and the high lines would have gone slightly upward due to the elimination of low numbers. Practically pure lines were established in the first generation. This, however, may be merely apparent, the lack of further effect of selection being due to the interference of somatic factors.

The male with 34 facets, giving rise to the brood 16f1–6, must be regarded as a mutant. This conclusion is based on the great difference between this male and its brothers in 16, the definite relation between the male parent and the female offspring, and the extremely low grade of the resulting females.

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In the Ba selections the results are very different and not so easily explained. Here also there must have been a downward shift as well as an upward shift. Disregarding the seventh generation for reasons already given, the following differences between the high and low lines are obtained: f1, 22; f2, 40; f3, 61; f4, 65; f5, 79; f6, 95. There is a gradual increase which becomes somewhat smaller as selection proceeds. If it were not for the interfering fluctuations the author's results would be much like those obtained by Zeleny and Mattoon. Even three additional generations of selection failed to produce pure lines.

In many respects the results obtained in these experiments resemble those obtained by MacDowell in the selection for extra bristles. The average of all the lines is raised or lowered but one can not predict within rather wide limits just what the offspring of any two parents are going to be like. This is clearly shown in Table II. and Fig. 6 in case of the matings d1f1–2 and d1f1–3. These matings were made with the same male but with females of 91 and 56 facets respectively. The average for the offspring from the higher female is considerably lower than that for the offspring of the lower female. In this case the unexpected results can not be explained on the basis that only a part of the character was observed. It is true that the counts were made only on one eye, but the difference between the two eyes falls within definite limits and is very small compared with the differences between the eyes of two flies. A comparable case would be the possibility of less than one extra bristle outside of the observed rows in the experiments of MacDowell. The large, parallel fluctuations of all the lines seem to indicate that there are environmental factors capable of almost doubling or cutting in half the facet numbers of flies of the same germinal constitution. The unexpected results must be regarded as being due to the fact that the hereditary factors are only a part of the total factor group controlling the facet number. When the environmental factors have been studied it may be possible to control them in such a way as to obtain uniform results. In that case more rigid selection will be possible.

The crosses made between the high and low lines show no evidence of sex-linkage. The offspring from both sets of matings

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are very nearly alike and there is no essential difference in the ratio of males and females. This agrees with the results obtained by MacDowell in his work on extra bristles. In the present experiments sex-linkage might have been expected from the fact that the ratio between male and female averages did not remain constant from generation to generation. The factors for converting the female means to the male standard in the seven generations of upward selection are successively 1.91, 1.73, 1.53, 1.40, 1.36, 1.60, 1.57. Those for the downward selections are 1.57, 1.86, 1.43, 1.79, 1.51, 1.46 and 1.40. But it is impossible to relate the differences in the offspring to differences in the parents.

The appearance of full-eyed males and heterozygous females in the stock and selected lines may be explained in two ways. It may have been due to the carelessness of the author in preparing the food, feeding and transferring the flies, or it may have been due to reverse mutation in the race.

The precautions taken by the author were not such as to exclude absolutely the possibility of the entrance of an egg here and there. But in spite of that fact the evidence is almost irrefutably against the theory of contamination. Perhaps it is well to state here in detail the author's methods. The precautions in regard to the preparation of food have already been given. It is sufficient to add that no larvæ ever appeared in the food jar. In all of these experiments the food was handled by means of an all-metal scalpel. This was used chiefly because it could be easily cleaned and could be kept absolutely clean. During the first two generations of VBa selections the author depended upon the fact that *Drosophila* does not as a rule lay eggs on a clean, dry, metal surface and merely kept the scalpel clean but did not heat it before using. In all succeeding work it was heated in an alcohol flame just before being used. All vials and bottles that had been used were boiled and rinsed in tap water and were then kept inverted on the table until they were again used. In the VBa selections no filter paper was used with the food. In the Ba selections the clean filter paper was kept in a table drawer and was removed only at the time of using. Fresh cotton for the plugs was also kept in table drawers.

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If old plugs were used over again they were either sterilized and kept in closed fruit jars or they were kept in closed jars for at least a week before using. Only plugs that had not been contaminated with food were used again.

But the evidence against the theory of contamination has little to do with the precautions taken in handling the material. In the first place all flies that appeared in the vestigial-winged lines had vestigial wings and all flies that appeared in the long-winged lines had long wings. The chief reason for choosing the vestigial-winged race for the selections was the fact that the second recessive could be used as a check in case of contamination. In all of the experiments no long-winged fly appeared in the vestigial-winged lines and no vestigial-winged form appeared in the long-winged lines. If the full-eyed and heterozygous flies had been due to contamination then the other characters should also have appeared, especially since for a long time the breeding vials of the two races were intermingled and treated as one lot. In the second place the author was handling no full-eyed, vestigial-winged flies at the time the first male appeared. It is true that Professor Zeleny had his stock of such flies in the same room at the time, but they were kept on a table about twenty feet from the one used for this work and the chances that a vestigial-winged fly will travel that distance are not very great. Finally, if the flies were due to contamination, full-eyed females as well as full-eyed males should have appeared; indeed, full-eyed females should have been more frequent than heterozygous females, but all females were heterozygous.

It is interesting to note that in all cases except one where full-eyed or heterozygous flies appeared in the selected lines it was in the high lines. The one exception was the heterozygous female in h3f2, a reverse selection from a low line.

More significant, however, is the fact that the females were always heterozygous. That means that if the change took place after fertilization only one chromosome in case of the females was affected. The males, of course, have only one chromosome bearing the factor. If the change occurred before fertilization, then it is probable that it appeared in the female germ cells. Had it taken place also in the male germ cells then

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heterozygous females should have appeared more frequently than full-eyed males and full-eyed females might in rare instances have appeared. If the change occurred indiscriminately in male or female then the proportion of heterozygous females to full-eyed males should be 3 to 1, but the ratio obtained is 5 to 6. The numbers obtained, however, are not large enough to be conclusive.

The fact of reverse mutation is very difficult to explain. It is hard to see how the germplasm can lose a factor and still potentially retain it and have it reappear later. So far as the present data go it is possible to explain the case under consideration in two different ways.

If the normal wild fly carries a limiting factor with respect to the facet number then it is possible by partial non-disjunction for the factor to pass from one chromosome of a pair to the other, giving one chromosome without a limiting factor and the other with a double limiting factor. The bar-eyed race of *Drosophila* may be derived from such a chromosome with two limiting factors or factor groups, the mate of the chromosome having been lost in the maturation of the egg. If then in the bar-eyed race a second non-disjunction again separates the two factors the result should be one chromosome with triple factors and one with a single factor, the latter giving rise to a full-eyed male or a heterozygous female. If the former passes into the egg it should give rise to a further reduction in the facet number, but it is possible that a fly with such a chromosome does not live. It is possible also that the male with 34 facets contained a chromosome with a triple reducing factor.

A simpler explanation is that of a reversible chemical change between two compounds one of which is more stable than the other. If the compound that forms the basis for the bar eye is the less stable then reversions are to be expected under certain conditions. But the fact that the change takes place only in the female is a strong argument in favor of the theory of partial non-disjunction.

Summary.

Selection was carried on in the vestigial-winged, bar-eyed stock for three generations and in the long-winged, bar-eyed

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stock for seven generations. The facets in the eyes of 9,000 flies were counted.

Selection in the vestigial-winged stock had to be discontinued on account of sterility and low production in the single lines.

The sterility and low production were not due to inbreeding. Sterility was not due to inability on the part of the females to lay eggs, nor does it appear to have been the final stage in the reduction of the number of offspring.

Selection in the vestigial-winged stock was effective for one generation but failed to produce further effects in the second and third generations. Return selections from the low lines were not effective.

A mutation appeared in a male in line 16. It was a reducing factor and was sex-linked. The complete sterility of the offspring of this male prevented further study of the character.

Selection in the long-winged stock was effective for six generations. In the downward selected lines most of the high flies were eliminated in the first three generations and no further effect of selection was noted. Return selections from the sixth generation, however, were still effective. The mean of the upward selected lines continued to increase at approximately uniform rate for six generations. The seventh generation must be disregarded because it was not an upward selection. The range in the facet number was not increased by selection. The results of these experiments indicate that the hereditary differences in this race of *Drosophila* are due to a large number of small factors.

Crosses made in the sixth generation between low and high lines indicate that the hereditary factors are not sex-linked.

In both the long-winged and the vestigial-winged lines the mean facet number was highly variable. This variability appeared to be due chiefly to changes in the temperature of the room, but may have been due also to other causes. The mean facet number of flies reared in vials as single lines was slightly higher than that of stock flies reared in bottles.

The mean facet number of the vestigial-winged flies was somewhat lower than that of the long-winged flies.

Six full-eyed males and five heterozygous females appeared in the stock bottles and the selected lines during these experiments. They must be regarded as reverse mutations.

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The reverse mutation can be explained on the theory of a reversible chemical reaction between two compounds one of which is more stable than the other. The present facts, however, favor an explanation on the basis of partial non-disjunction.

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MITOSIS AND AMITOSIS.

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It was once supposed that all forms of nuclear division were of the type which is now called amitosis in which the nucleus undergoes simple constriction. After the discovery of the complicated method of nuclear division now known as mitosis and the recognition of its very general occurrence in plants and animals, the doubt was expressed whether amitosis ever occurs as a normal process; its occurrence in pathological and degenerating cells is undoubted. However, the number of cases in which nuclear constriction is known to occur regularly in certain tissue cells is too great to warrant the belief that it is always a pathological phenomenon.

The only question of present importance in connection with this form of nuclear division is as to its bearing on the chromosome theory of heredity. If amitotic division of the nucleus, followed by division of the cell body, ever occurs in the germ cell cycle or in cleavage cells undergoing normal differentiation it would go far to disprove the "individuality" or persistent identity of chromosomes and consequently the chromosome theory of inheritance, since it is scarcely possible that individual chromosomes can be equally divided and their halves accurately distributed to daughter cells by the process of amitosis. The majority of cytologists at the present day concur in the opinion that amitosis does not normally occur in embryonic or germ cells and that when it does occur in such cells the ensuing development is abnormal. On the other hand some investigators maintain that amitosis occurs regularly and predominantly in the genesis of the germ cells and also in the blastomeres and embryonic cells of several species of animals and plants and for this reason among others they reject the chromosome theory. Child in particular has maintained this position in a series of papers (1907–1911) dealing primarily with the cestode *Monezia*,

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but secondarily with several other animal forms ranging all the way from cœlenterates to birds.

Among other recent writers who have maintained a similar view are Hargitt (1904, 1911) on amitosis in the cleavage of the eggs of cœlenterates, Patterson (1908) in blastoderm cells of the pigeon, Glaser (1908) in embryonic cells of *Fasciolaria*, Jordan (1908) in spermatogonial divisions of *Aplopus*, Nathansohn (1900) in *Spirogyra*, Wasielewski (1902, 1903) in the root tips of *Vicia*, Gurwitsch (1905) in the blastomeres of *Triton*, Maximow (1908) in embryonic tissues of the rabbit, Jörgensen (1908) in the oögenesis of *Nephelis*, Moroff (1909) in the eggs of copepods, Knoche (1910) in the insect ovary, Nowikoff (1910) in bone and sinew cells of the young mouse, and Foot and Strobell (1911) in the ovary of *Protenor*—all of whom hold that amitosis may occur as a normal process in germinal and embryonic cells. Several of the authors named as well as R. Hertwig (1898), Lang (1901), Calkins (1901), Herbst (1909), Godlewski (1909) and Konopacki (1911) hold that there is no principal distinction between mitosis and amitosis and that they may both occur without interfering with normal processes of differentiation.

On the other hand, this view is contested by Boveri (1907) and Strasburger (1908) on general grounds and is not confirmed by the experiments of Häcker (1900) and Schiller (1909) on *Cyclops* eggs subjected to ether, nor by the experiments of Němec (1903) who repeated the work of Wasielewski on the root tips of *Vicia* subjected to chloral hydrate and reached the conclusion that the supposed amitoses are really modified mitoses. Richards (1909, 1911) and Harmon (1913) carefully and laboriously repeated Child's work on cestodes and found no evidence of amitosis in germ cells or cleavage cells, while on the other hand there was abundant evidence of mitosis. Child (1911) has reëxamined the question and reaffirms in the main his former opinion, and this has been confirmed by Young (1913), whose general conclusions, however, are so reactionary and even revolutionary that they cannot be accepted without further confirmation.

Boveri (1907) has said that if Child would establish his contention he must prove (1) that the binucleate condition which

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he finds is actually due to a division, (2) that a portion of the cytoplasm is cut off around each of these nuclei, (3) that the cells arising in this manner again divide mitotically and have the normal number of chromosomes; in his opinion not one of these proofs has been furnished. The binucleate or multinucleate condition, he adds, may be due to an incomplete fusion of chromosomal vesicles after mitosis, as Rubaschkin (1905) has shown to be true for the blastomeres of *Triton*. Boveri concludes, therefore, that the individuality theory has not been shaken by this work on amitosis. There is no doubt that constricted or lobulated nuclei are sometimes found in germ cells or embryonic cells; the critical question is whether these are stages in the amitotic division of the nucleus and if so whether the cell bodies divide and are capable of normal development. It is evident from a large number of observations on a great variety of objects that constricted nuclei are not in themselves sufficient evidence that amitosis is occurring, for many such nuclei of living cells have been observed to lose the constriction and later to divide by mitosis. And even if such constricted nuclei actually divide, the chromosomal constitution of the cell could remain normal provided the cell body did not divide following the amitotic division of the nucleus.

As a matter of fact, amitotic division of the nucleus is rarely followed by division of the cell body. Macklin (1916) has recently studied amitosis in living tissue cells of the embryo chick and, although he was able to follow the process of nuclear division in successive stages in one and the same cell, in no case was such amitotic division of the nucleus followed by division of the cell body. Such amitotic division was sometimes followed by mitosis and in such cases the two or more nuclear vesicles in a cell gave rise to chromosomes which formed a single equatorial plate. Macklin concludes, therefore, that "there is nothing in nuclear amitosis opposed to the chromosome hypothesis"—with which opinion I entirely agree.

Unfortunately it is rarely possible to study all stages of nuclear and cell division in normal living cells, not only because of the indistinctness with which one sees what is going on inside the cell, but also because of the difficulty of keeping cells alive and

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normal for a sufficient length of time under conditions of observation; furthermore, failure to observe amitosis in one hundred such cells would still leave it possible that it might occur in a second hundred. There is, however, a method by which the occurrence or non-occurrence of amitosis can be determined with great certainty and this is in the study of identical cell divisions in hundreds and thousands of individuals. This is possible only in the two maturation divisions which are so peculiar that they can always be distinguished from each other and from all other divisions, and in those early cleavages where the lineage of every cell is known and its method of division can be observed in thousands of different cases. If in every such dividing cell one sees mitosis only, it can be concluded that this is the invariable method of division in these cells. It is a significant fact, which Child himself admits, that in no such case have the nucleus and cell ever been seen to divide by amitosis, whereas in hundreds and thousands of cases they have been seen to divide by mitosis.

Nevertheless, in these very cells one frequently observes lobulated, constricted or bipartite nuclei which might be regarded as stages in amitosis were it not for the fact that the study of the lineage of these cells shows that they invariably divide by mitosis and that the peculiar shapes of the nuclei referred to are due to modifications of normal mitosis. Such constricted or bipartite nuclei occurring in cells the lineage of which was unknown would naturally be mistaken for cases of amitosis and it is very significant that the reports of amitosis in embryonic cells have been invariably in cells of unknown lineage.

This paper is based on a study of modified mitosis in the maturation and cleavage of the eggs of the marine gasteropod, *Crepidula plana*. A variety of nuclear forms are figured and described which resemble more or less closely stages of amitosis and yet it is perfectly certain that these nuclei divide only by mitosis, and the manner of origin of these pseudo-amitotic forms is plainly due to modifications of regular mitotic processes. These modifications were produced by subjecting dividing cells to abnormally high temperatures or to sea-water of abnormal densities, but other similar modifications are produced by many other abnormal conditions such as pressure, centrifugal force,

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carbonic acid and various other chemical substances (Conklin, 1912). These different types of modified mitoses may be classified under the following heads: (1) Scattering of chromosomes and their failure to unite into a single nuclear vesicle, (2) amitotic or mitotic division of the nucleus without division of the cell body and the subsequent division of such binuclear or polynuclear cells, (3) separation of chromatin and achromatin and formation of cytasters, (4) persistence of nuclear membrane and formation of chromatic connections between daughter nuclei.

I. Scattering of Chromosomes and Their Failure to Unite Into a Single Nuclear Vesicle.

In the late anaphase of normal mitoses the chromosomes of the daughter plate stick together so that when the individual chromosomes begin to take in achromatic substance and to swell up into chromosomal vesicles the whole plate is converted into a mulberry-like mass which later becomes a single nuclear vesicle either by the fusion of the separate chromosomal vesicles or by their closer approximation. There is a growing body of evidence that in certain cases at least these closely appressed chromosomal vesicles do not completely fuse with one another but preserve their individuality (Bonnevie, 1908, for *Ascaris* and *Allium*; Vejdovsky, 1912, for *Ascaris* and *Decticus*; Wenrich, 1916, for *Phrynotettix*; Richards, 1917, for *Fundulus*). In other cases, when it is not possible to recognize a distinct vesicle for each and every chromosome, maternal and paternal chromosomes may form more or less distinct vesicles (Häcker, 1895, for *Cyclops*; Conklin, 1901, 1902, for *Crepidula*).

In certain abnormal conditions, and especially by means of temperatures higher than normal and by hypertonic sea-water, the division and separation of daughter chromosomes may be delayed or stopped and the chromosomes scattered along the length of the spindle (Figs. 9, 10, 29). After the chromosomes have reached the poles of the spindle, they may be separated from one another and remain scattered more or less widely in the cell. If the temperature is not too high (34°–35° C.) each separate chromosome will then swell up to form a separate vesicle, or if two or more chromosomes are in close contact they may form a single vesicle of larger size (Figs. 7, 9–12).

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If the temperature is a little higher (37° C.) the chromosomes may stick together in an irregular mass and be drawn to the surface of the cell, probably by the complete contraction of the fibers which anchor the spindle to the peripheral layer of the protoplasm (Figs. 13, 14, 17). In the latter instance the chromosomes do not swell up and become vesicles, but remain permanently small and densely chromatic. Owing to some change, probably in the peripheral layer of each chromosome, caused by the high temperature, the chromosomes are unable to take up achromatin. Such chromosomes and the cells containing them never recover and never go further in development, although the cells do not immediately undergo degeneration and to all appearances remain alive for twenty-four hours or longer.

Of a piece with this scattering of chromosomes is the failure of all the chromosomal vesicles to unite into a single vesicle. All degrees of fusion of chromosomal vesicles may be found from those which remain wholly separate to those which are united into a single spherical vesicle. Other things being equal, the size of a nuclear vesicle varies according to the number of chromosomal vesicles which enter into it. In this way arise nuclear forms which have been called "fragmented nuclei," "multinuclear cells," "bipartite," "lobulated" and "elongated" nuclei (Figs. 1–12, 19–28), although it is evident in the case of *Crepidula* that these nuclear forms have not arisen by constriction or fragmentation of an originally single nucleus.

These partial nuclei formed by incomplete fusion of the chromosomal vesicles were first called "karyomeres" by Fol. That they are partial and not entire nuclei is shown by the fact first established by Boveri that in the next following mitosis each karyomere gives rise only to the same number of chromosomes as entered into it and not to the full number of chromosomes characteristic of the species; this fact I have repeatedly confirmed in my studies on *Crepidula*. And that such karyomeres are due to the failure of chromosomes to unite into one vesicle and not to the amitotic division of a single vesicle is shown by the following considerations:

1. They are most numerous in the telophase of division (Figs. 7–12) where the chromosomes are sometimes widely scattered

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and where every chromosome may give rise to a separate chromosomal vesicle (Fig. 7). As the cell passes into the resting phase, these vesicles fuse together more or less completely, giving rise to vesicles of varying sizes. Other things being equal, the later the stage in the resting period the smaller the number of separate vesicles. If the separate vesicles were formed by division of an originally single vesicle, exactly the reverse would be the case.

2. The presence of centrospheres and spindle remnants in many cases shows conclusively that division has taken place by mitosis, and the position of these structures indicates the location of the mitotic figure (Figs. 8–12 *et seq.*).

3. The elongation of daughter nuclei in the position of the plate of daughter chromosomes of normal division figures indicates that such elongated nuclei are formed by the partial fusion of the chromosomes of the daughter plate (Figs. 4–6).

These considerations make it absolutely certain that these peculiar nuclear forms are due to a partial or incomplete union of chromosomes into a single nuclear vesicle in the final phase of mitosis; they represent modified mitosis and not amitosis. And it is practically certain that many of the cases of so-called amitosis described by several of the authors mentioned above are of this same character.

It is not easy to determine exactly the mechanism by which these modifications have been produced. As already mentioned they may be caused by abnormally high temperature (Figs. 1–12) or by hypertonic sea-water (Figs. 19–29). It is well known that when the daughter chromosomes approach the poles of the spindle they are normally closely crowded together and it seems probable that this is due to linin connections within the spindle or between the chromosomes. In the development of the daughter nucleus each chromosome absorbs achromatic material from the cytoplasm and becomes a vesicle with chromatic walls. The material thus absorbed is probably chiefly water though it doubtless contains dialyzable proteins and other substances which may be assimilated into the chromatin and linin of the nucleus. It is probable that there is a real membrane surrounding each chromosome (Conklin, 1902, 1912) and that the

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absorption of surrounding substances by the chromosome takes place through this membrane by a process of dialysis. The nuclear vesicle is most nearly spherical in form when it is largest and when its contents are most fluid in character irregular or lobulated nuclei are usually smaller and the nuclear contents more dense (Figs. 1–6). Therefore the modifications of mitosis, which prevent the union of chromosomes into a single vesicle, act by modifying the walls of these vesicles so that they do not readily unite and so that they do not readily absorb fluid from the cytoplasm.

II. Amitotic or Mitotic Division of Nuclei Without Division of Cell Body and Subsequent Division of Such Binuclear or Polynuclear Cells.

In the cleavage of *Crepidula* a binucleate or multinucleate cell is invariably due, so far as I have observed, to a failure of chromosomal vesicles to unite into a single vesicle. In other cases, however, it is plain that elongated, constricted and bipartite nuclei have resulted from the constriction of a single nuclear vesicle. In addition to numerous cases which have been described by other investigators, I have myself studied such cases in the egg-follicle cells of *Gryllus* (Conklin, 1903), the liver cells of *Porcellio* (Conklin, 1897), as well as in some muscle cells and connective tissue cells. In none of these cases, however, is division of the nucleus followed by division of the cell body. Although every follicle cell of *Gryllus* and every liver cell of *Porcellio* shows the nucleus in some stage of amitosis, and although many of these cells contain two entirely separate nuclei, in no single instance have I ever seen a division of the cell body separating these nuclear halves.

Since these cases of amitosis occur in differentiated tissue cells it may be assumed that the nuclei are not active in the further differentiation of these cells; on the other hand their metabolic activity is great and the nuclei are undoubtedly concerned in this activity. It has been assumed that the division or lobulation of such nuclei favors metabolic activity by increasing the surface of the nuclei and bringing them into closer relation to all of the cytoplasm of the cell, and this is probably true. Espe-

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cially in the case of elongated muscle cells the division of the nucleus and the distribution of the daughter nuclei along the length of the fiber must facilitate interaction between nucleus and cytoplasm, and the same is true, though perhaps to a smaller extent, in gland cells and egg-follicle cells.

Dahlgren and Kepner (1908) hold that the very numerous amitoses in the striated muscle cells of the embryo fish, *Catostomus*, may be followed in some instances by the division of the muscle cell. But since the plane of nuclear division is always transverse to the fiber, while the plane of cell division is always longitudinal, it could not be affirmed that the cell divisions in this case correspond to the nuclear divisions. But even if amitoses may be followed by division of the cell body in these cases, it must not be forgotten that all these cells are fully differentiated and according to the chromosome theory the nucleus has already performed its differentiating functions while its further function in the fully differentiated cell is probably purely trophic.

Many observations and experiments demonstrate that the nucleus is concerned in the two functions (1) of differentiation or regulation and (2) of metabolism; the work of Gruber (1886), R. Hertwig (1898), Heidenhain (1894), Henneguy (1896), Conklin (1902) *et al.* indicates that the chromosomes or basichromatin are particularly concerned with the former, the oxychromatin or achromatin with the latter. It is a significant fact that chromosomes divide only by mitosis and Boveri (1908) has shown that a complete set of chromosomes is necessary to normal differentiation. On the other hand oxychromatin and achromatin divide only by amitosis even in cases of mitotic division of the chromosomes. The significance of these facts seems to have been missed not only by those who maintain the equivalence of mitosis and amitosis, but also by Weismann and his followers who assumed that in embryonic differentiation there is a differential division of chromosomes and a "disintegration of the germ plasm" with segregation of particular factors in particular cells. For if individual chromosomes differ in hereditary potencies, every division by amitosis must be a differential one, while on the other hand every typical mitotic

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division is non-differential so far as the chromosomes are concerned.

Since it is usually impossible to see outlines of individual chromosomal vesicles in the resting nucleus, it is not possible to determine whether the constrictions and lobulations of amitosis merely separate whole vesicles from one another. If they do the number of chromosomes arising from each of these vesicles in subsequent mitosis should be the same as in the case of karyomeres formed by the failure of vesicles to unite in the resting stage.

It is probable from the work of Boveri (1907) and of Macklin (1916) that when amitotic division of the nucleus is followed by mitosis, each nuclear vesicle gives rise to a fraction only of the normal number of chromosomes and that all the nuclear vesicles in a cell taken together give rise to no more than the normal number. Furthermore, the work of Boveri demonstrates that there is no return to the normal number of chromosomes when once a cell contains an abnormal number. Each nuclear vesicle produced by amitotic division is therefore a karyomere, in every way comparable to those produced by incomplete fusion of chromosomal vesicles; it is a fragment of a nucleus and not an entire nucleus, and this is equally true whether all the karyomeres lie within a single cell, as is usually the case, or whether in some rare instances they may be distributed to separate cells. In *Crepidula* it matters not how many karyomeres there are in a cell, if there are two and only two centrosomes all the chromosomes come together into a single plate and there is a normal division and distribution of each of these chromosomes to the daughter cells.

Therefore in considering the significance of amitosis it is of the utmost importance to know whether the constriction of the nucleus is followed by a division of the cell body; if it is not, amitosis is not a permanent nuclear division at all but merely a temporary separation of karyomeres which come together again into a unit structure at the next mitosis. It is a significant fact that in most instances amitotic division of the nucleus is not followed by division of the cell body.

In this connection it is worth while to compare with the

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conditions just described those which obtain when mitosis is not followed by division of the cell-body. In a former paper (Conklin, 1912) I have described such cases at some length and need not here go into details. In brief, if the daughter nuclei and centrosomes lie so far apart in the undivided cell body that they do not interfere, in subsequent mitoses every one of these mitoses may be entirely normal and development may be typical except that no differentiation ever appears between the halves of the undivided cell. On the other hand, if the daughter centrosomes lie near together in the undivided cell body so that they interfere we get tripolar or tetrapolar figures with irregular distribution of chromosomes and usually with the formation of several karyomeres of varying sizes depending upon the number of chromosomes entering into them. Such multipolar mitoses in *Crepidula* are rarely followed by division of the cell body so that at every succeeding mitotic period the number of centrosomes and chromosomes in this undivided cell body are approximately doubled (Figs. 26–29). Of course such cells with abnormal numbers of chromosomes and centrosomes never develop normally. Normal differentiation depends upon the regular distribution into separate cells of daughter centrosomes and chromosomes as well as of different cytoplasmic substances.

III. Separation of Chromatin and Achromatin and Formation of Cytasters.

The behavior of the chromatic and achromatic parts of the nucleus in hypertonic and in hypotonic media throws a certain amount of light on the constitution of the normal nucleus and on the behavior of these nuclear constituents during normal mitosis. When resting nuclei are subjected to hypertonic solutions (*e. g.*, 2–4 per cent. NaCl in sea-water) the chromatic portion of the nucleus contracts into a small dense mass leaving the achromatic portion as large as ever (Figs. 32, 43, 48). It looks as if the chromatin had undergone complete "plasmolysis" whereas the volume of the achromatin had not been affected at all. The membrane or boundary of this achromatin remains full and unshrunken, which would presumably not be the case if this outline represented a real plasma membrane. The

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shrinkage of the chromatin on the other hand probably indicates that it is surrounded by a plasma membrane, or more likely that each chromosome is so surrounded.

When resting nuclei are subjected to hypotonic solutions the entire nucleus becomes slightly swollen and less deeply chromatic, which indicates that the chromatic parts of the nuclei take up water, probably through the chromatic nuclear membrane or the chromosomal membranes.

The achromatic membrane, or rather boundary, is regularly spherical in resting stages but during mitosis it disappears or else becomes so indefinite and irregular in outline that it is difficult to recognize. However the achromatic substance of the nucleus together with some of the denser portion of the cytoplasm constitutes the amphiaster with its nuclear spindle and astral radiations. In hypertonic solutions the amphiaster is sharply set off from the surrounding cytoplasm (Figs. 35–36), due as I believe in the main to the condensation of its substance and the elimination from it of the more fluid cytoplasm. In this process of condensation the astral radiations are largely drawn into the central part of the figure but portions of these radiations may become isolated from the amphiaster and thus form independent condensation centers. These have a radiating structure and are typical cytasters, but unlike those described by Wilson they do not in *Crepidula* divide nor form the poles of true mitotic figures. My observations on the origin and nature of these cytasters (Conklin, 1912) entirely agree with those of Konopacki (1911) and in the main with the observations of Mead (1898) and Morgan (1899).

Cytasters appear best developed during periods of mitosis when the achromatin is distributed in the astral radiations (Figs. 33–35) but they are also abundant in eggs after the maturation divisions and before the first cleavage (Figs. 31, 32) and in such cases one can frequently see that they lie along the radiations of the maturation aster (Fig. 31).

During prolonged resting periods, especially when the eggs are in strong salt solutions, cytasters are replaced by faintly staining vesicles (Figs. 37–42) which appear to contain achromatic nuclear material. These vesicles are surrounded by a delicate achromatic

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membrane and they resemble R. Hertwig's "nuclei without chromatin." They are found chiefly in the position of the previous spindle remnants and along the lines of astral radiations. Usually the largest of these achromatic vesicles are in close proximity to the dense mass of chromatin, which in these cases does not become vesiculated. In some instances there is a single elongated achromatic vesicle in each daughter cell which occupies the position of the interzonal fibers of the spindle (Figs. 43–48) and which may inclose the dense mass of chromosomes at the spindle ends. Such conditions give the appearance of an amitotic division of the nuclear vesicle, but the presence of centrosomes, mid-bodies and in some instances of spindle fibers and astral radiations as well as of chromosomal plates (Figs. 46, 47) clearly shows that these divisions are true mitoses in which the chromosomes have been prevented from absorbing achromatin while the latter has formed a definite boundary or membrane separating it from the cytoplasm.

Just as the size of a central aster is reduced by the presence of numerous cytasters or parasitic asters which surround it, so the size of the chromatic nuclear vesicle is inversely proportional to the volume of the achromatic vesicles in the cell. It seems practically certain that the chromosomal vesicles and consequently the entire chromatic portion of the nucleus grow by the absorption of this achromatic substance. When nuclei are large they contain much achromatic substance and at the same time there are no cytasters or achromatic vesicles in their vicinity; when they are small and densely chromatic there may be cytasters in the cell during the periods of mitosis, or achromatic vesicles during resting periods. "The cytasters are therefore, in my opinion, isolated portions of archiplasm (achromatin plus spongioplasm) derived in large part from escaped achromatin, which take the aster form during mitosis and the vesicular form during resting periods" (Conklin, 1912, p. 543).

IV. Persistence of Nuclear Membranes and Formation of Chromatic Connections Between Daughter Nuclei.

It has generally been assumed that one of the strongest evidences that amitosis had occurred in any given case was to be

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found in the incomplete separation of daughter nuclei or in chromatic connections between them. Thus Gurwitsch (1905) has figured and described the division of a blastomere of a centrifuged *Triton* egg in which two nuclei, connected by a chromatic thread, are dividing by mitosis. The chromatic connection is taken as proof positive that the nuclei had divided by amitosis and Godlewski (1909) in a general review of this subject, after dismissing as doubtful many other cases in which amitosis had been reported as occurring in normal development, falls back upon this case described by Gurwitsch as one of the strongest evidences in favor of the view that amitosis may occur in normally differentiating cells.

But chromatic connections between nuclei are not to be taken as positive evidence that those nuclei have divided by amitosis, for these connections may be the result of incomplete or atypical mitoses. Anything which retards or prevents the separation of daughter chromosomes may lead to the scattering of chromosomes along the spindle or to their elongation into threads and consequently to the formation of chromatic connections between daughter nuclei. Häcker found such connections in etherized eggs of *Cyclops* and such connections are present also in *Crepidula* eggs subjected to high temperatures (Figs. 6, 8), to hypertonic sea-water (Fig. 29), and to hypotonic sea-water (Figs. 49–60). It is especially in the last named experiments that chromatic connections between daughter nuclei are most frequently seen and they merit a detailed description.

The eggs shown in Figs. 49–54 were placed for one hour in sea-water diluted with two volumes of fresh water and were then returned to normal sea-water for four hours before being fixed; those shown in Figs. 55–60 were placed for two hours in sea-water diluted with one volume of fresh water and were then left in normal sea-water for fourteen hours. In all of these cases the centrosomes divided normally and approximately normal spindles were formed but the separation of chromosomes and the formation of daughter nuclei were atypical. In Figs. 9, 29, 49 and 50 the scattering of chromosomes is shown in some of the spindles but more notable than this is the stretching of chromosomes into long threads some of which run from one

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pole of the spindle to the other. When chromosomes are merely scattered throughout the cell or along the spindle they usually give rise to chromosomal vesicles wherever they lie, as is shown in Figs. 7, 16, 26, etc., but when in addition they are stretched into elongated threads chromatic connections are left between daughter nuclei.

In diluted sea-water the chromosomes show a tendency to stick together into masses and to stretch out into long threads instead of dividing and moving to the two poles of the spindle. This is probably due to the fact that the linin basis of the chromosomes is modified so that the latter do not preserve their usual shapes and do not separate normally in division. The nuclear membrane also frequently remains chromatic and in such cases may persist throughout mitosis (Figs. 49–53, 55, 59, etc.). Evidently some of the chromatin which usually enters into the formation of the chromosomes is left in these cases at the periphery of the nucleus. Since the nucleus is composed of chromosomal vesicles more or less completely united this result might conceivably be due to the swelling and bursting of some of these vesicles at the nuclear periphery.

When mitosis is halted in the prophase of the third cleavage the centrosomes separate and a spindle is formed in the usual manner but the nuclear membrane persists and the entire nucleus becomes pear-shaped (Fig. 49, cell D), or unequally constricted (Figs. 51, 52), the smaller portion corresponding to the micromeres containing almost all of the deeply staining chromatin in the form of chromatic threads, while the larger portion belonging to the macromeres contains faintly staining threads and granules. These two portions of the constricted nuclei are approximately proportional in size to the nuclei of the normal micromeres and macromeres, although in cell D, Fig. 49, and cells C and D, Fig. 52, the division wall between the micromeres and macromeres has not formed. The fact that these two portions of the constricted nuclei are proportional in size to the cells to which they belong even when the division wall between those cells has not formed is difficult to explain. Generally the size of a nucleus is proportional to the volume of cytoplasm in which it lies (Conklin, 1912) because the chromosomal vesicles

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absorb substance from the cytoplasm in their growth, but in this case the entire nucleus has undergone constriction and the cell body has not.

The aggregation of chromatin on the side of the nuclear vesicle on which the spindle lies causes it to collect on the animal pole side of the nucleus in Figs. 49–51 and on the side away from the animal pole in cell D, Fig. 54. In every instance the chromatin collects at that pole of the nucleus which is next to the centrosome or spindle; this is a general phenomenon which has been remarked by R. Hertwig in *Actinospherium*, by Calkins in *Noctiluca*, Conklin in *Crepidula*, etc. In this connection one recalls that in many protozoa the nuclear membrane persists throughout mitosis the spindle being within the nuclear vesicle. The chromosomes divide and separate as in typical mitosis but the nuclear membrane and vesicle constrict as in amitosis. In the ciliata the micronucleus divides by mitosis, the macronucleus by amitosis. In metazoa also the chromosomes alone divide by mitosis, or the splitting of the chromatic thread, while the division of all other nuclear constituents is a mass division.

The peculiar form of nuclear division which is caused by the stretching out of the chromosomes and the persistence of the nuclear membrane superficially resembles amitosis but is really a modified form of mitosis. Nuclei which have divided in this peculiar manner go on dividing by mitosis when they are returned to normal sea-water. Thus cells A and C, Figs. 49 and 50, cells A and B, Figs. 52 and 53, and all the macromeres in Fig. 54 are shown dividing by mitosis. These are typical mitotic figures though the number and arrangement of chromosomes may be atypical. In all of these cases the dividing nucleus of the macromere is connected with the nucleus of the micromere by a chromatic strand and it is particularly noticeable that this strand always runs to the plate of chromosomes in the macromeres and usually to the outer side of this plate. Since the original chromatic connection united the nuclei by their distal poles (away from the centrosome) the fact that when these nuclei divide the connection runs to the proximal pole (toward the centrosome) indicates that the chromatin changes position within the nuclear vesicle, being drawn to the proximal pole of the nucleus (Fig. 54, *D*).

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Again the way in which the chromatic connection between nuclei unites with the chromosome plate (Figs. 53, 54) shows that this connection is actually composed of chromosomal substance though it has been so modified that it does not give rise to separate chromosomes nor does it show any tendency to divide or split, as normal chromosomes do, into daughter chromosomes. Although it is not possible to count the number of chromosomes in these plates it is evident that it varies in different cases and that in general it is less than normal, which is what would be expected if the chromatic connections represent a number of spun-out chromosomes. Furthermore the fact that these connections do not swell up to form vesicles as normal chromosomes do, indicates that the chromosomal substance of which it is composed has undergone some significant change.

Still later divisions of the cells connected by these chromatic strands are generally abnormal, as is shown in Figs. 55–60. The eggs shown in these figures were subjected to diluted sea-water during the third cleavage and were then returned to normal sea-water where some of the cells have undergone the fourth, fifth and sixth cleavages. In many cases the chromosomes at each of these cleavages have been stretched out into chromatic connections between daughter nuclei and since all of these persist a complicated network of such connections is present between nuclei of successive generations (Figs. 55, 56, 58, 60); at the same time the number of chromosomes in a plate is in many instances greatly reduced (Fig. 55).

The same types of modifications produced by diluted sea-water persist after the eggs have been returned to normal sea-water and in all of these cleavages, if one saw only the end result, the chromatic connections would seem to indicate that the nuclei had divided by amitosis. However a study of various stages in this process shows conclusively that this is not the case but that all of these divisions are modified forms of mitosis.

V. Conclusions.

1. The modern revival of interest in amitosis is due to a reaction against the chromosome theory. If nuclear and cell divisions ever take place by amitosis in normally developing sex

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cells and embryonic cells it would deal a fatal blow to that theory. The occurrence of amitosis in fully differentiated tissue cells or in cells which do not undergo division would not affect the chromosome theory.

2. When direct division of the nucleus occurs it is rarely if ever accompanied by division of the cell body. The individual nuclear vesicles or karyomeres are not whole nuclei but fragments of a nucleus and when the cell actually divides these karyomeres combined form the typical number of chromosomes which unite into a single spindle and divide in the typical manner, as recently shown by Macklin.

3. Many apparent cases of amitosis are merely modified mitoses of which the following forms are described in this paper:

(*a*) The scattering of chromosomes and their failure to unite into a single nuclear vesicle.

(*b*) Mitotic division of the nucleus without division of the cell body and the consequent formation of binucleate or polynucleate cell.

(*c*) The failure of daughter chromosomes to pull apart in the spindle and the consequent formation of chromatic connections between daughter nuclei.

(*d*) The persistence of the nuclear membrane with division of the chromosomes by mitosis and of the nuclear vesicle by constriction.

4. There is not a single wholly conclusive case in which amitosis has been shown to occur in the division of normally differentiating cells. Therefore the attempts to disprove the chromosome theory in this way have failed.

5. Mitosis and amitosis are fundamentally unlike. Mitosis is the one and only method of bringing about equal division and distribution of the chromatic material of the nucleus. Amitosis is not a genuine divisional phenomenon at all but merely a means of increasing the nuclear surface and of distributing nuclear material throughout the cell, comparable to nuclear lobulation, fragmentation or distribution. These two processes are not equivalent or even comparable nor may one of them be converted into the other.

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Description of Figures.

Figs. 1–60 represent whole eggs of *Crepidula plana* which had been fixed, stained and mounted. All figures were drawn with camera lucida under Zeiss apochromatic objective 3 mm. Hom. Im., Comps. ocular 4. As drawn they represent a magnification of 333 diameters; in the process of reproduction they have been reduced about one third.

Figs. 1–18 represent eggs which had been subjected to increased temperature.

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Explanation of Plate I.

Fig. 1. No. 1174(2). 37°, ½ hr., during second maturation; then kept at room temperature (ca. 27°) for 3 hrs. The first polar body is shaded by transverse lines; the sperm nucleus is shown at the left; the other nuclei are karyomeres formed by the scattering of chromosomes of the second maturation mitosis.

Fig. 2. No. 1175(2). 33°, ½ hr., during first cleavage; room temperature (ca. 27°) 3 hrs. The imperfect fusion of the karyomeres (gonomeres?) gives an appearance of amitosis to each of the nuclei.

Fig. 3. No. 1176(2). 34°–35°, ½ hr., during second cleavage; room temperature (ca. 23°) 3 hrs. Karyomeres formed by scattering of chromosomes of second cleavage.

Fig. 4. No. 1173(2). 37°, ½ hr., beginning of third cleavage; room temperature (ca. 27°) 3 hrs. Many karyomeres formed by scattering of chromosomes of third cleavage. Traces of centrospheres are shown in the cells.

Fig. 5. No. 1176(2). 34°–35°, ½ hr., during third cleavage; room temperature (ca. 23°) 3 hrs. Karyomeres formed by scattering of chromosomes of third cleavage. In one quadrant a micromere was formed, in the other quadrants cell division was suppressed. Traces of centrospheres as in preceding.

Fig. 6. No. 1173(2). 37°, ½ hr., during anaphase of third cleavage; room temperature (ca. 27°) 3 hrs. In two quadrants micromeres have formed. Daughter nuclei are elongated in the greater dimensions of the chromosome plates, the latter having fused together into irregular masses.

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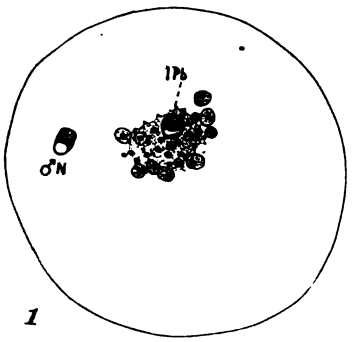

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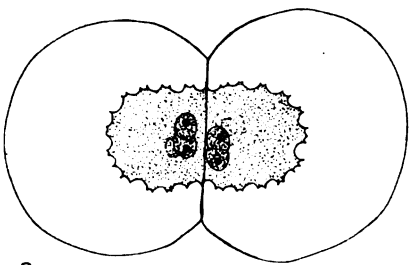

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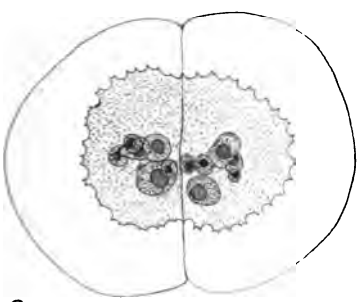

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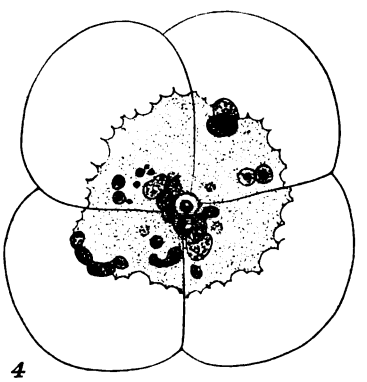

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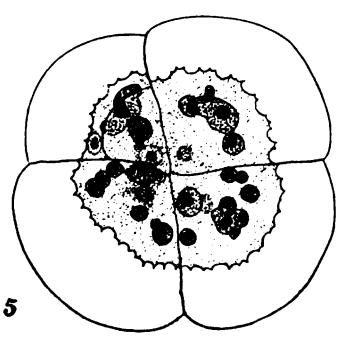

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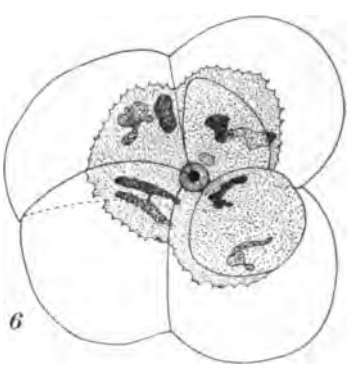

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Explanation of Plate II.

Fig. 7. No. 960. Ca. 35°, 4 hrs., during third cleavage. The chromosomes have scattered and formed numerous chromosomal vesicles.

Fig. 8. No. 1176(2). 34°–35°, ¼ hr., during anaphase of third cleavage; room temperature (ca. 23°) 3 hrs. Many nuclei are lobed or irregular; nuclei are most abnormal in the quadrant in which a micromere did not form, and in which mitosis was not so far advanced at the time of the experiment, as in the other quadrants.

Fig. 9. No. 1176(1). 34°–35°, ¼ hr., during fourth cleavage of macromeres. In three quadrants the chromosomes are scattered in the spindles, in the fourth are chromosomal vesicles. The nuclei and centrosomes in the first set of micromeres are in a resting stage and are absolutely normal.

Fig. 10. No. 1176(1). 34°–35°, ¼ hr., during fourth cleavage. Chromosomal vesicles are present in one of the macromeres, and chromosomes are more or less scattered in all micromeres of the first set.

Fig. 11. No. 1176(2). 34°–35°, ¼ hr., during fourth cleavage; room temperature (23°) 3 hrs. The nuclei formed after the division of the first set of micromeres are lobed and irregular; the scattered chromosomes in the macromeres have formed many karyomeres. This figure shows an egg like Fig. 9 after being kept three hours at normal temperature.

Fig. 12. Same slide as preceding. Many lobulated nuclei and karyomeres in the cells derived from the first set of micromeres; nuclei in second set of micromeres and in macromeres are nearly normal. This figure shows an egg like Fig. 10, after being kept three hours at normal temperature.

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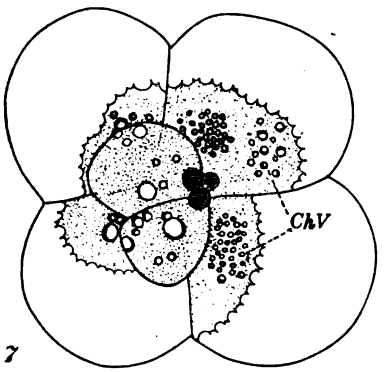

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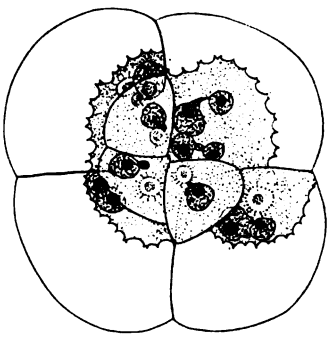

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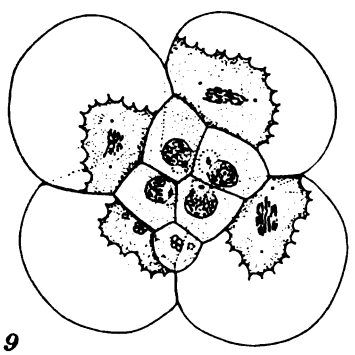

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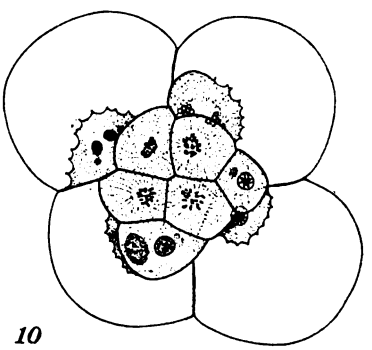

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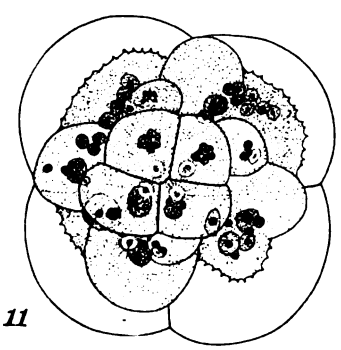

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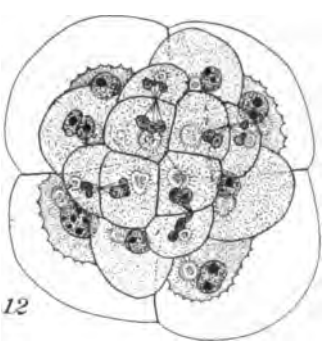

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Explanation of Plate III.

Figs. 13–18 were subjected to temperature so high (37°), or for so long a period (35° for four hours) that further cell division was stopped in almost all cases, though the protoplasm remained transparent and apparently alive.

Fig. 13. No. 1171(2). 37°, ½ hr. during first cleavage; room temperature (ca. 25°) 15 hrs. Astral areas are large and distinct; chromosomes are widely scattered or clumped in two principal masses outside the astral areas and at the surface of the egg; chromosomes do not become vesicular.

Fig. 14. Same slide as preceding; subjected to heat during second cleavage, and showing results similar to preceding.

Fig. 15. No. 960. Ca. 35°, 4 hrs., during resting 2-cell stage. The plasma is much contracted and vacuolated and the chromatin of the nuclei is in the form of hollow spheres, which look like chromosomal vesicles.

Fig. 16. Same slide as preceding; heated during second cleavage mitosis; plasma shrunken and chromosomes in the form of vesicles.

Fig. 17. No. 1171(2). 37°, ½ hr., during fourth cleavage; room temperature (ca. 25°) 15 hrs. Chromosomes in the macromeres are clumped together at the surface of the egg, and are outside the plasma areas. There is a triaster in one of the micromeres, while the others appear normal.

Fig. 18. Same slide as preceding; 24-cell stage. All centrospheres instead of being at the surface, as in normal eggs, are at the deeper ends of the cells next to the spacious segmentation cavity.

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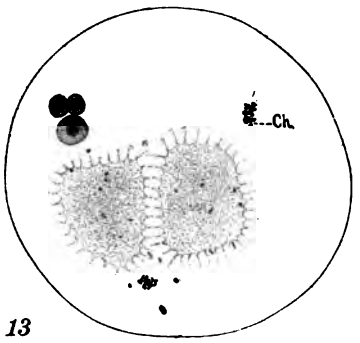

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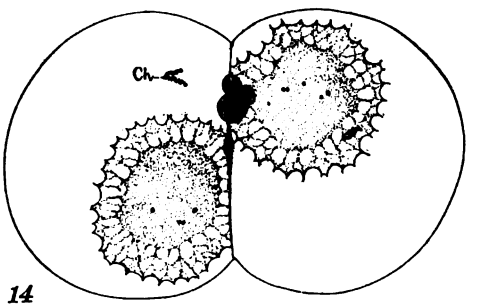

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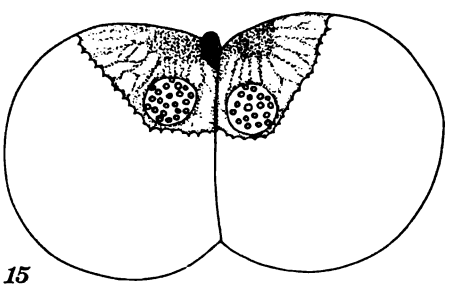

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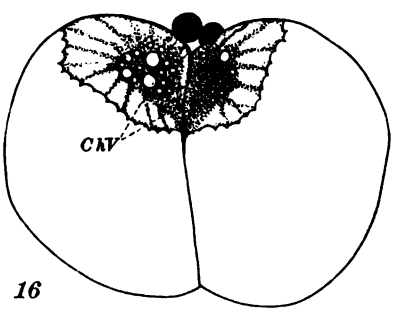

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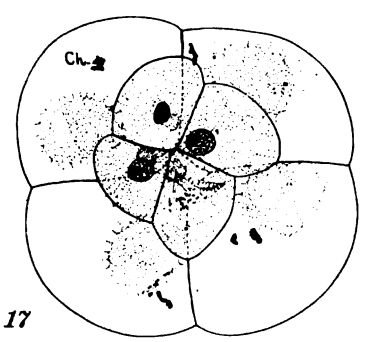

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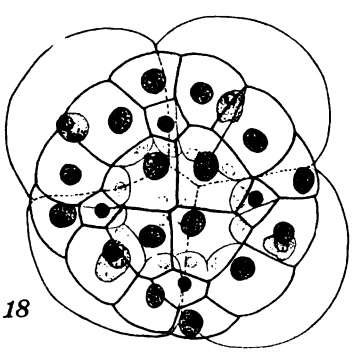

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Explanation of Plate IV.

Figs. 19–48 (with exception of Fig. 30) represent eggs which had been subjected to hypertonic solutions.

Fig. 19. No. 822. 2 per cent. NaCl, 16 hrs., normal sea-water 8 hrs. Numerous karyomeres were formed after maturation and before the first cleavage, presumably during the first cleavage mitosis.

Fig. 20. Same slide as preceding. Karyomeres formed at the close of the first cleavage by chromosomes failing to unite. Centrospheres (*s*) unusually large.

Fig. 21. No. 837. $\frac{3}{4}$ per cent. KCl 9 hrs.; normal sea-water 35 hrs. Karyomeres irregular in size, shape and distribution.

Fig. 22. Same slide as preceding, with similar karyomeres.

Fig. 23. No. 822. 2 per cent. NaCl 16 hrs.; normal sea-water 8 hrs. Second cleavage abnormal, with karyomeres in two of the cells.

Fig. 24. No. 814. 2 per cent. NaCl 1 hr.; normal sea-water 17 hrs. Third and fourth cleavages abnormal, with karyomeres in most of the cells.

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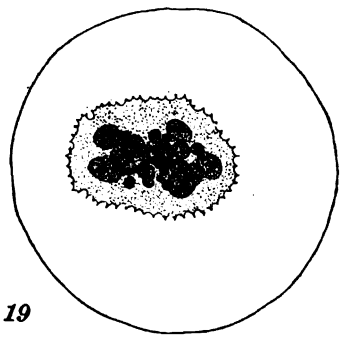

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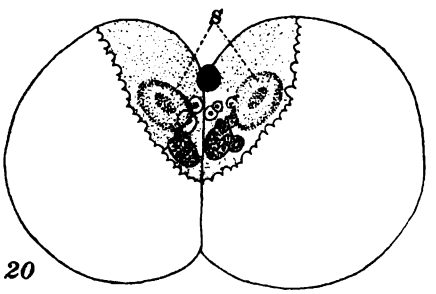

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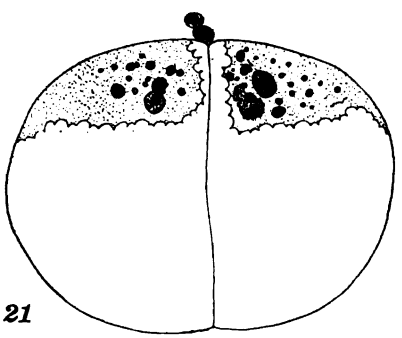

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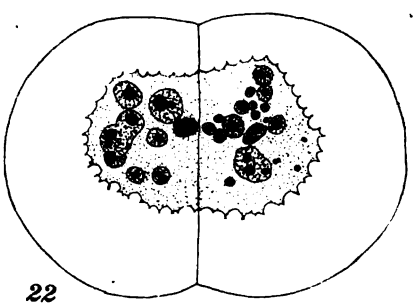

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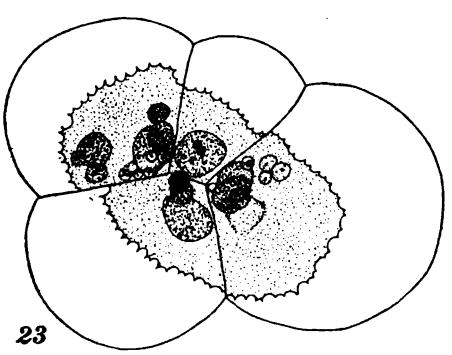

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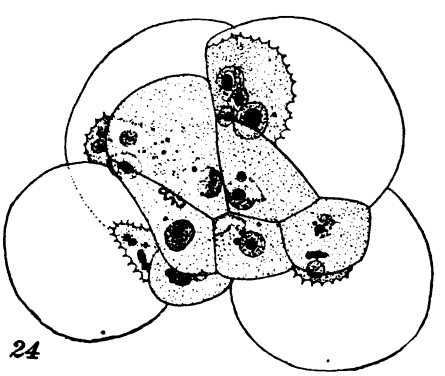

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Explanation of Plate V.

Fig. 25. No. 927. 2 per cent. NaCl 16 hrs., at close of third cleavage; normal sea-water 24 hrs. Numerous karyomeres in the cells.

Fig. 26. No. 867. 8 per cent. $MgCl_2$ $\frac{3}{4}$ hrs., during third cleavage; normal sea-water $6\frac{1}{2}$ hrs. Numerous karyomeres in resting cells; in dividing cells, polyasters and scattered chromosomes.

Fig. 27. No. 972, same slide as Fig. 25. Karyomeres in all resting cells; polyasters and scattered chromosomes in dividing ones.

Fig. 28. No. 971. 2 per cent. NaCl 16 hrs., during third cleavage; normal sea-water 12 hrs. Similar to preceding.

Fig. 29. No. 828. 1 per cent. NaCl 2 hrs., during third cleavage; normal sea-water $6\frac{1}{2}$ hrs. Polyasters and chromatic connections between daughter nuclei.

Fig. 30. No. 956. Sea-water diluted with equal parts of fresh water 2 hrs.; normal sea-water 36 hrs. The nuclei in yolk-containing cells show many karyomeres.

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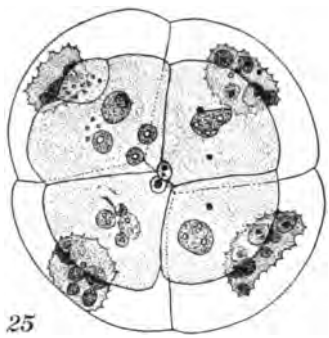

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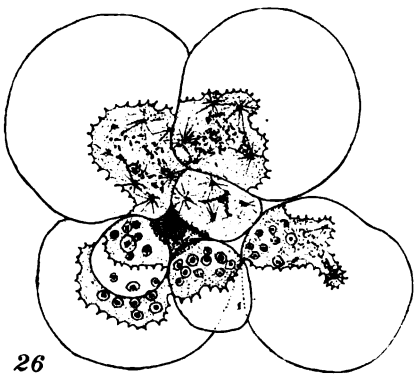

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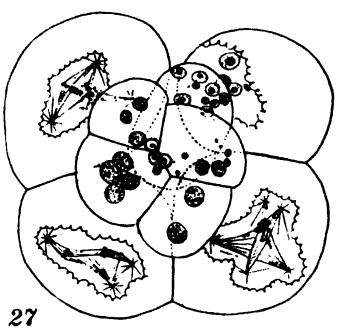

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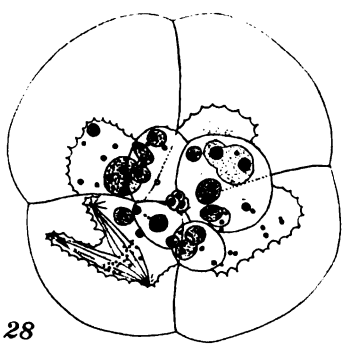

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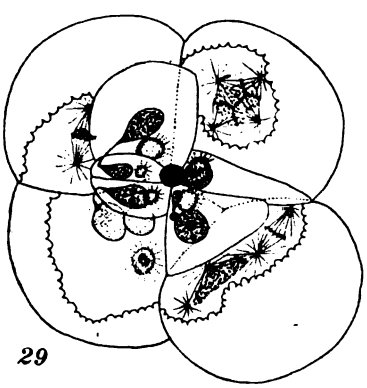

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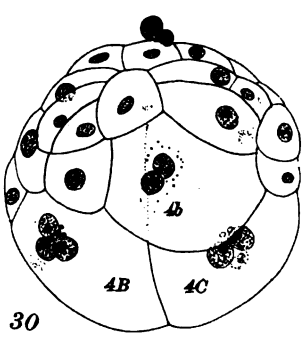

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Explanation of Plate VI.

Fig. 31. Normal egg showing cytasters sometimes present in eggs at the stage between the close of the maturation divisions and the beginning of the first cleavage. These cytasters are local aggregations of plasma along the lines of astral radiations.

Figs. 32, 34, 35. Eggs from Exp. No. 805. 2 per cent. NaCl 4 hrs. Numerous cytasters are shown in different cell stages and division phases; in every case these cytasters are local aggregations of plasma along the lines of astral radiations, the remaining plasma being gathered around the nuclei or spindles.

Figs. 33, 36. No. 998(3). 1 per cent. NaCl 5 hrs. The plasma is concentrated around the spindles, only small portions being left along the astral radiations in the 2-cell stage and none in the 4-cell stage. No cytasters are present after the 2-cell stage.

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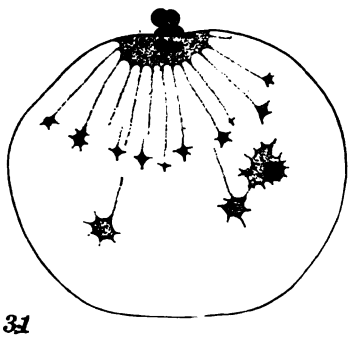

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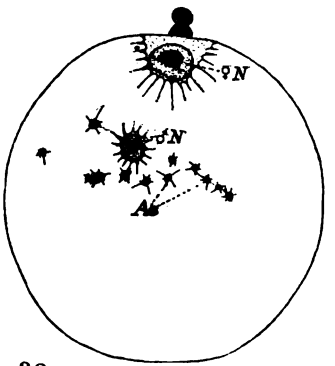

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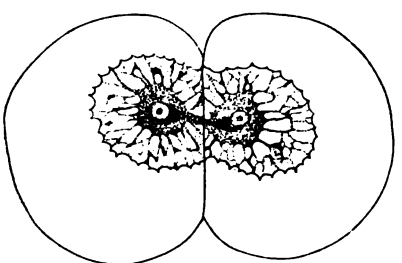

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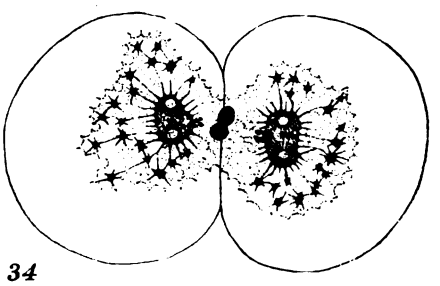

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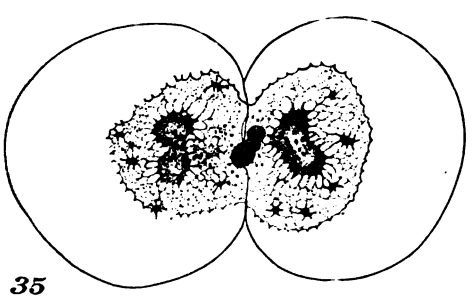

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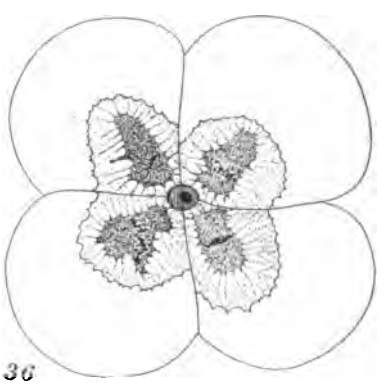

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Explanation of Plate VII.

Figs. 37–42. Various eggs from one experiment, viz., No. 809. 2 per cent. NaCl 15½ hrs. All figures show the chromatin in a densely chromatic mass and in all cases except Fig. 37, where the egg nucleus consists of several karyomeres, all the chromatin of each nucleus is aggregated into a single mass. On the other hand the achromatin consists of vesicles of different sizes which are more or less widely scattered, though a large achromatic vesicle is usually found adjoining a chromatic mass. This achromatin has not been squeezed out of the chromatic mass by the action of the salt solution, but the chromosomes of the daughter nuclei have been prevented from absorbing achromatin by the action of the salt solution.

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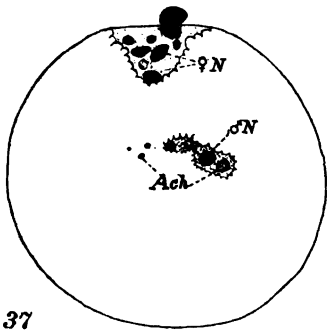

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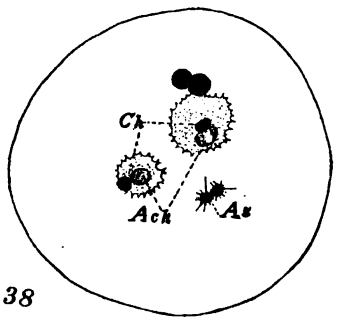

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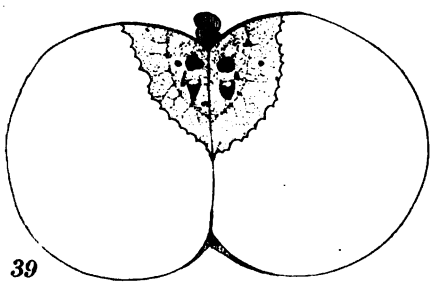

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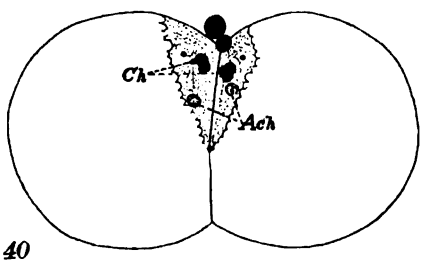

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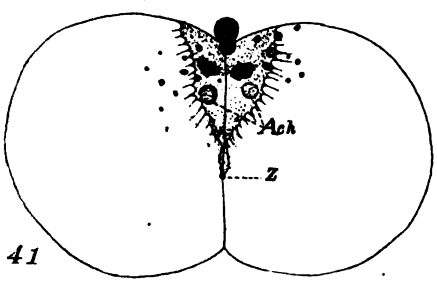

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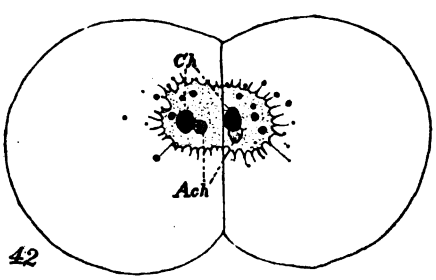

42

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Explanation of Plate VIII.

Figs. 43–48 are different eggs from the same experiment, viz., No. 810. 3 per cent. NaCl 15½ hrs. All the eggs were in the anaphase or telophase of the second cleavage at the time they were placed in the salt solution, and in all cases the chromosomes have remained in the form of a dense chromatic plate or mass which has not become vesicular. In Fig. 45, the spindle remnants between the chromosomal plates has become vesicular; in Figs. 46 and 47, the spindle area has become an elongated achromatic vesicle, within which lies the dense chromatic mass; in Figs. 43, 44, 48 the achromatic vesicle has become more nearly spherical in outline, while the chromatic mass is not quite so dense as in earlier stages. These figures show that when the chromosomes are prevented from absorbing achromatin and becoming vesicular nuclei, the achromatin of the spindle region may become a vesicle by the formation of a delicate achromatic membrane around itself.

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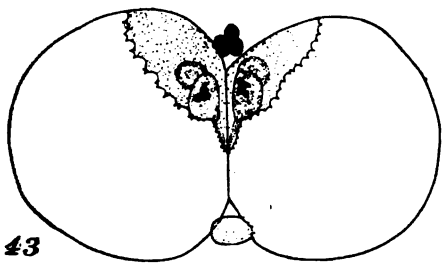

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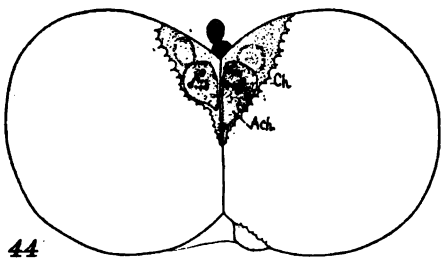

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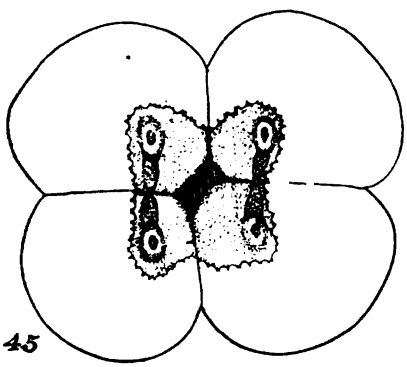

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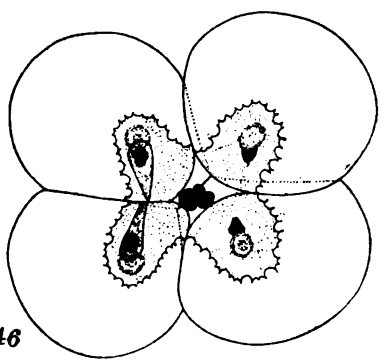

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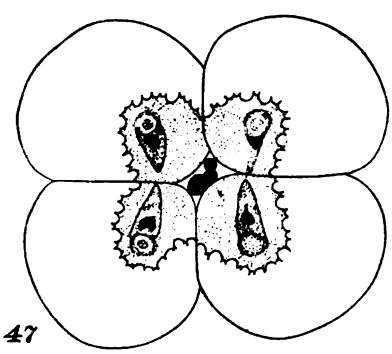

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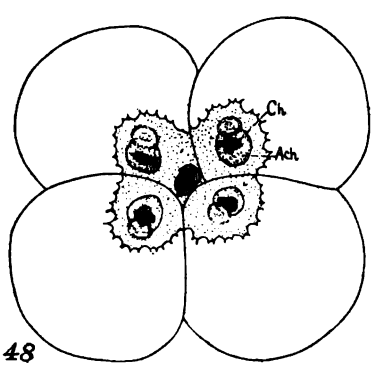

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Explanation of Plate IX.

Figs. 49–60 represent eggs which had been subjected to diluted sea-water.

Figs. 49–54. No. 859. Sea-water 1 part, fresh water 2 parts, 1 hr.; normal sea-water 4 hrs. Eggs were treated with this diluted sea-water during the third and fourth cleavages, thus causing a scattering or stretching out of chromosomes along the spindle and the formation of chromatic connections between daughter nuclei. In cell D, Fig. 49, the division was stopped in the prophase, the nucleus being pear-shaped with the chromatin chiefly in the narrow upper end of the pear. It is significant that the long axis of the pear is in the direction of the spindle axis and that if the constriction were to separate the neck from the body of the pear, the daughter nuclei thus formed would be of approximately the same size as in normal eggs.

Fig. 51 shows more advanced stages of a similar process in which the chromatin is chiefly in the smaller, upper nuclei, the achromatin in the larger, lower ones.

Figs. 49 and 50 show certain nuclei in which the nuclear membrane remains intact though the chromosomes are arranged along a line or spindle connecting the two centrosomes.

Figs. 52–54. Eggs in which the third cleavage took place by a modified form of mitosis which left chromatic connections between daughter nuclei, and yet typical spindles for the fourth cleavage are present in some of the macromeres.

Fig. 52. In all four quadrants of this egg nuclear division at the third cleavage took place by modified mitosis, the daughter nuclei remaining connected by chromatic threads; in only two quadrants were micromeres formed, and the macromeres of these quadrants are now dividing by mitosis.

Figs. 53, 54. All daughter nuclei are connected by chromatic threads. Chromatin aggregates on the side of the nucleus next the centrosome (Fig. 54, *D*) and the chromatic connections between daughter nuclei run to the outer sides of the nuclei and spindles in the macromeres; the latter may be approximately normal, though the spindles may be out of proper position and the chromosomes more or less scattered.

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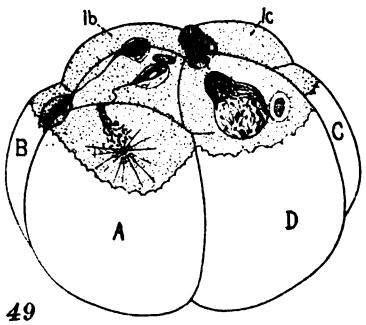

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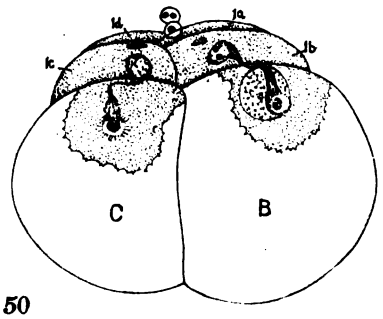

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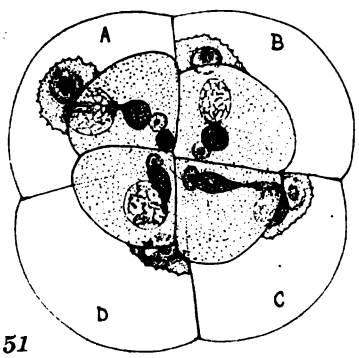

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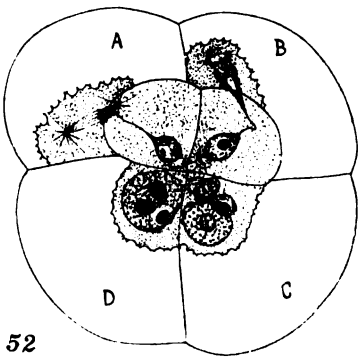

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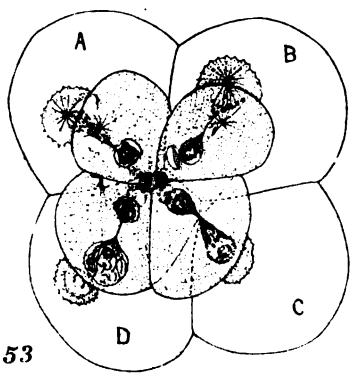

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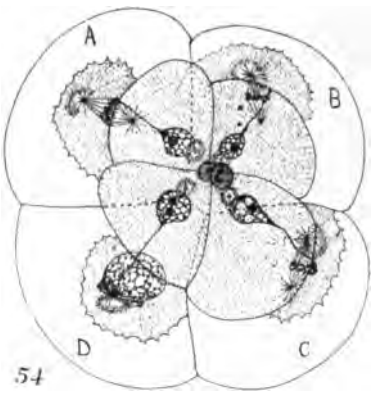

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Explanation of Plate X.

Figs. 55–60. All from one experiment, No. 858. Eggs in 2–4-cell stage were left in sea-water diluted with equal parts of fresh water for two hours; then in normal sea-water fourteen hours. Remains of chromatic connections which were formed during the third cleavage are seen in Figs. 55, 56, 60. Other chromatic connections which were formed in later cleavages after the eggs had been returned to normal sea-water are shown in Figs. 57–60.

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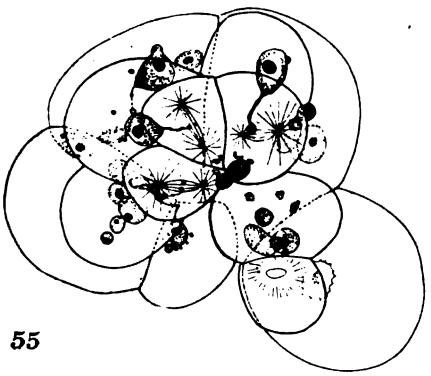

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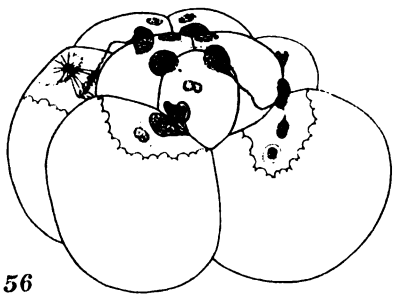

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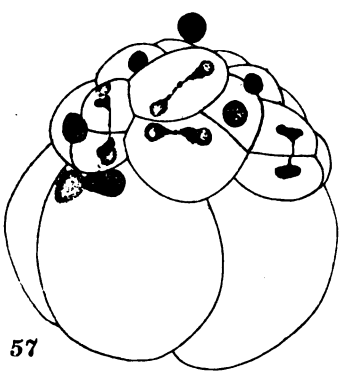

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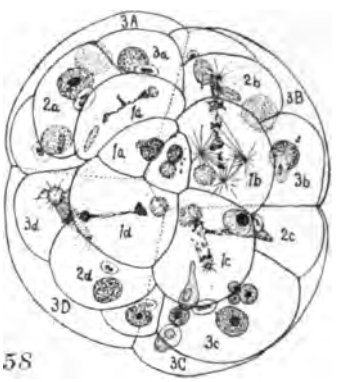

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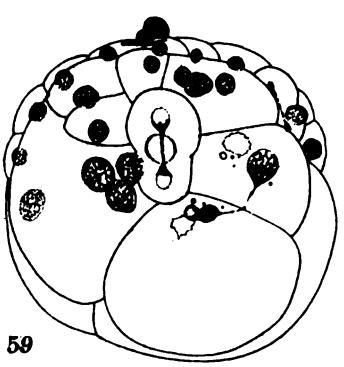

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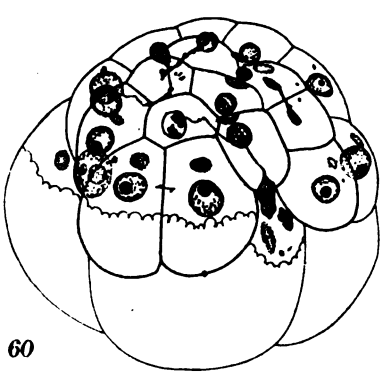

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THE INFLUENCE OF GENERAL ENVIRONMENTAL CONDITIONS ON THE PERIODICITY OF ENDOMIXIS IN PARAMECIUM AURELIA.

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OSBORN ZOÖLOGICAL LABORATORY, YALE UNIVERSITY.

(Twelve figures.)

It is clear from the evidence submitted in previous papers that there are normal, minor, periodic fluctuations (rhythms) in the rate of reproduction of *Paramecium*,[1] and that at the low point in the division rate between two rhythms there normally occurs an intracellular reorganization process (endomixis).[2] The data previously presented also show that in *Paramecium aurelia* endomixis occurs, generally speaking, at intervals of about four weeks or about fifty generations, and that the synchronism of the process in different lines of the same race under identical conditions is remarkably exact.[3] But, as was stated incidentally, on the basis of our experience in working out the cytological phenomena of endomixis: "It is possible to retard or hasten the occurrence of the process by the character of the culture medium. For example, it may occur a few days earlier in animals not supplied daily with fresh culture fluid than in the regular lines." [4] Such being the case it is important to determine the influence of environmental conditions on the duration of the rhythms and the occurrence of endomixis.

The present paper comprises chiefly a study of the effects of what may be termed general changes in the culture conditions,

[1] Woodruff and Baitsell, "Rhythms in the Reproductive Activity of Infusoria," *Journ. Exper. Zoöl.*, XI., 4, 1911.

[2] Woodruff and Erdmann, A Normal Periodic Reorganization Process without Cell Fusion in *Paramecium*," *Journ. Exper. Zoöl.*, XVII., 1914; Erdmann and Woodruff, The Periodic Reorganization Process in *Paramecium caudatum*," *Journ. Exper. Zoöl.*, XX., 1916; Woodruff, "Rhythms and Endomixis in Various Races of *Paramecium aurelia*," BIOL. BULL., XXXIII., 1917.

[3] Cf. Woodruff and Erdmann, 1914, Tables 1, 2, 3.

[4] Woodruff and Erdmann, 1914, p. 485.

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such as markedly different culture media and temperatures on rhythms and endomixis. It seemed important to obtain a more definite background of knowledge of the influence of what perhaps may be called normal environmental changes before attempting to study the influence of, for example, specific chemical agents on the process.

It is assumed in the present paper that the reader is familiar with the earlier work on *Paramecium* which has been published from the Yale Laboratory.

Material and Methods.

The organisms employed in the work were from pedigreed cultures of *Paramecium aurelia*. Some of these had been under culture conditions for long periods—one for more than 5,000 generations—while others were started with this work in mind. Each of the five races used was started originally with a 'wild' individual which was secured from a locality far removed from that of the others, so that representative diverse material was afforded. The early life history of each of these cultures has been presented in connection with other work and the reader is referred to these papers for further details.[1]

All the organisms studied have been carried in pedigreed subcultures isolated from the respective main cultures of the various races, and since the method of conducting such cultures has been described many times in earlier papers it need not be repeated in detail here. Suffice it to say that the method involves the isolation of one or more animals from each line of every subculture practically every day and in addition, for the work in hand, the preservation and cytological study of some of the stock animals left over at the time of the daily isolations. In this way the occurrence of endomixis has been determined.

The main cultures have been carried on the 'varied' culture medium which we have found for ten years so favorable in breeding *Paramecium*.[2] This consists of infusions of vegetable and animal debris collected from time to time from laboratory

[1] Cf. especially Woodruff, Biol. Bull., XXXIII., 1917.

[2] Woodruff: "The Life Cycle of *Paramecium* when Subjected to a Varied Environment," *American Naturalist*, XLII., 1908.

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aquaria, ponds, etc., and, of course, thoroughly boiled and allowed to attain room temperature before being used. Some of the subcultures directly involved in the experiments have been bred on this medium. Others have been bred on the beef-extract medium which we have employed in other work on *Paramecium*,[1] or on other media which will be described in connection with the individual experiments.

The subcultures which have been the basis of the present work may be tabulated as follows:

SUBCULTURES.

A (from Main Culture I) Oct. 15, 1914, to Feb. 12, 1916. (485 days.)
4675th to 5592d generations.

AE (from IE)[2] Oct. 15, 1914 to Aug. 12, 1915. (Twice restarted during the 300 days.)
4637th to 5079th generations.

O (from III) Oct. 15, 1914 to Nov. 20, 1915. (Once restarted during the 400 days.)
17th to 775th generations.

B (from IV) Jan. 8, 1915 to Jan. 14, 1916. (372 days.)
3d to 609th generations.

M (from V) July 17, 1915 to Feb. 23, 1916. (222 days.)
3d to 550th generations.

W (from VI) Aug. 12, 1915 to Jan. 14, 1916. (152 days.)
3d to 302d generations.

The data are presented chiefly by graphs of the division rate of the various subcultures. These are plotted by averaging the daily rate of division of the several lines of the respective subcultures and then again averaging this for five-day periods. The figures 1, 2, 3 represent divisions and 10, 20, etc., indicate the number of the five-day periods. An *E* shows that endomixis was observed during the five-day period. Inclusion of a part of the curve within brackets indicates that the cells were not studied cytologically during this time. Cf. Figs. 1 and 7. Since so much depends on these graphs and the five-day periods which they comprise it may be well to repeat a statement made in a previous paper:[3]

[1] Woodruff and Baitsell, "The Reproduction of *Paramecium aurelia* in a 'Constant' Culture Medium of Beef Extract," *Journ. Exper. Zoöl.*, XI., 1, 1911.

[2] *IE* is a subculture isolated from Main Culture I. in October, 1913.

[3] Woodruff and Erdmann, *Journ. Exper. Zoöl.*, 1914, p. 477.

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"The five-day period was adopted in the presentation of our results because this was the method of constructing the

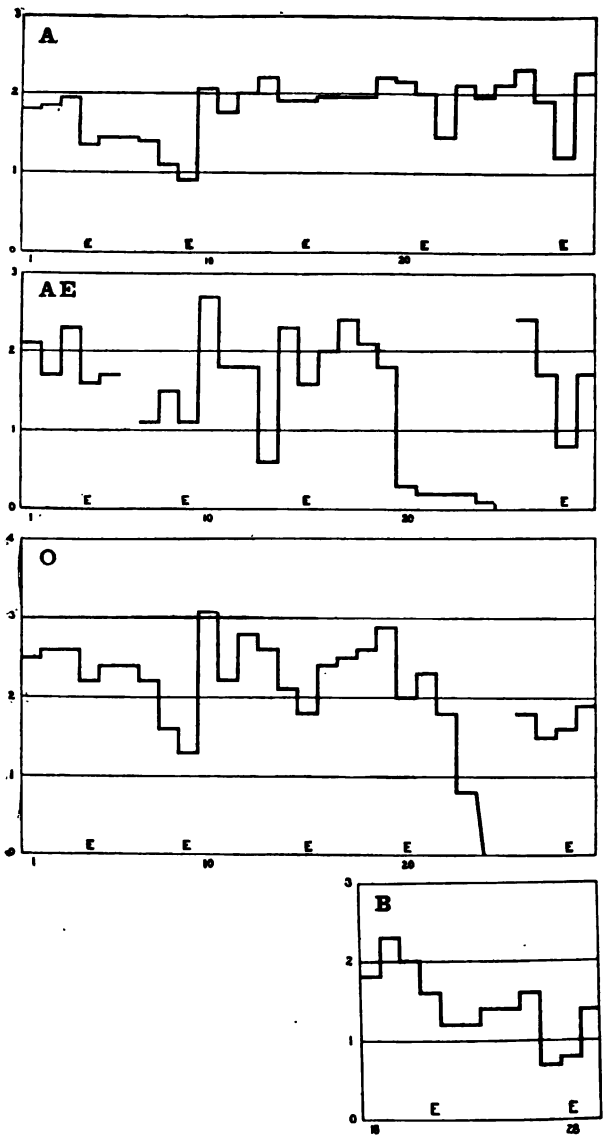

FIG. 1.

graph emphasized in the original study of rhythms in this culture.[1] It is realized, of course, that a five-day period is

[1] Woodruff and Baitsell, *Journ. Exper. Zoöl.*, XI., 1, 1911.

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largely an arbitrary one and that the ideal graph would present the momentary changes in the metabolism of the cell. Data for such a curve being absolutely impossible to secure, it might seem at first glance that the daily record of division would approach most nearly to this ideal condition. As a matter of fact, the twenty-four-hour period is as arbitrary as the five-day period when it is considered that this is a long period when compared with the metabolic changes in the cell and that the daily record, made at approximately 11 A.M., would merely give the divisions actually completed during the previous twenty-four hours. For example, let us assume that, at the time of isolation, two animals are present, representing one division during the previous twenty-four hours. The record for that day is one division. One animal is then isolated and it divides within an hour and each of the resulting cells again divide twice before the next isolation. The record for this second day is three divisions, thus the record for the two days shows a different division rate for each day, *i. e.*, one division against three divisions, whereas a more true, but not a perfect, picture of the state of affairs is given by the statement that four divisions occurred in forty-eight hours. One might follow this argument to its logical conclusion and assume that the best method of presentation would be to average for considerable periods, *e. g.*, 10 or 30 days, but this obviously would tend to obliterate any fluctuations in the rate which are not of relatively long duration. The adoption of the five-day period was made in recognition of both of these contingencies, and it was of a duration particularly well suited to show the effect of the process on the reproductive rate, because the process extends over about nine cell divisions or a period of about six days. Consequently the effect of the process makes itself evident in the five-day plot. Certain apparent irregularities in the coincidence of the phenomena are, from an actual study of all the data at hand, clearly due to the fact that the five-day period is not ideal."

Experiments—Series I.

The experiments of Series I. may be outlined as follows:

A. Study of the periodicity of rhythms and endomixis in

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different races of *Paramecium aurelia* when bred under the same *varied* culture conditions.

B. Study of the periodicity of rhythms and endomixis in

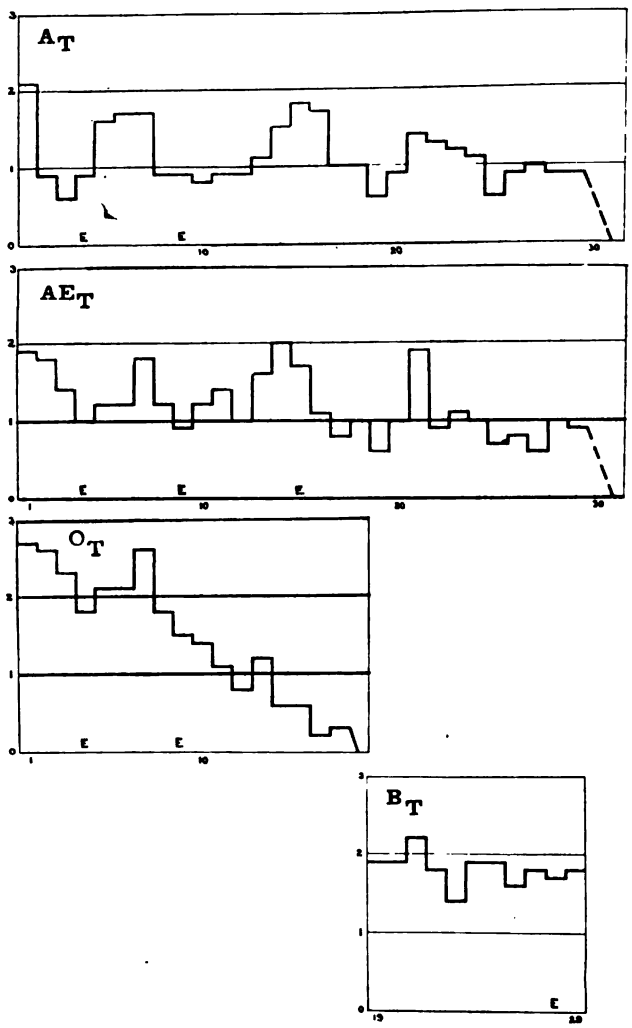

FIG. 2.

different races of *Paramecium aurelia* when bred under the same *constant* culture conditions.

C. Study of the periodicity of rhythms and endomixis in the *same* races of *Paramecium aurelia* when bred under the *varied* and under the *constant* culture conditions.

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These experiments were carried on from October 15, 1914, to March 10, 1915; a period of 145 days. The subcultures employed were *A*, *AE*, and *O* throughout the work and *B* from its isolation on January 8, 1915, to the end. Each of these subcultures represents a different race of *Paramecium aurelia*, except *A* and *AE*, both of which were originally derived from the same stock, Main Culture I., about 1,000 generations before.

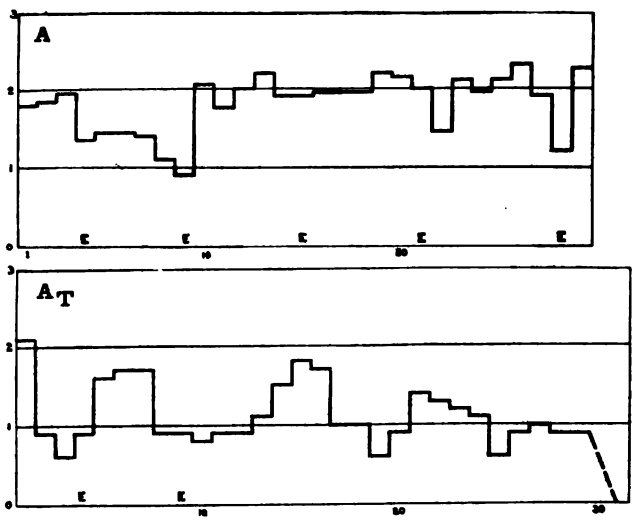

FIG. 3.

All of these subcultures were subjected to a varied culture medium and to the ordinary fluctuation in room temperature.

Sister subcultures (designated *At*, *AEt*, *Ot* and *Bt*), were isolated line by line from the above cultures at the start of the experiment and were subjected to a constant culture medium of beef extract. The temperature was maintained relatively constant at about 26° C. in a thermostat.

A.

Study of the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred under the same *varied* culture conditions.

Fig. 1 presents the graphs of the division rate of subcultures *A*, *AE*, *O* and *B* throughout this experiment. A study of the

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figure shows that *A* underwent endomixis at periods Nos. 4, 9, 15, 21 and 28. *AE* showed endomixis at period 4 and then was lost by an accident. A new *AE* was started by isolating line by line from *AEt* (which had been branched from it 25 days before and subjected to the constant culture conditions).

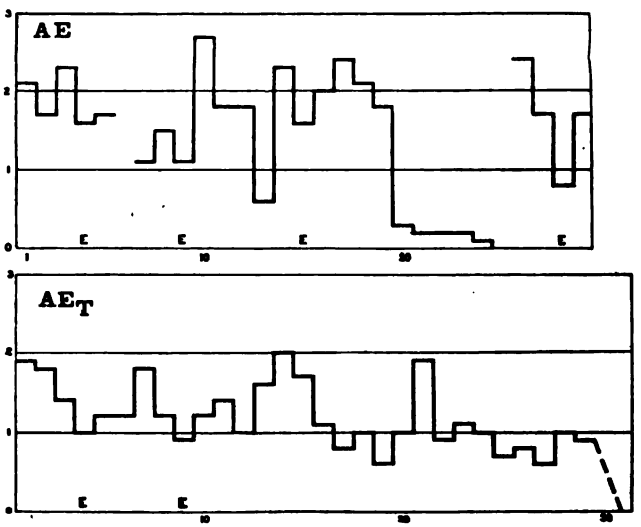

FIG. 4.

AE then showed endomixis at the periods 9 and 15 and died out in period 25 without again undergoing endomixis. Still another *AE* was isolated immediately from *AEt* and had endomixis during the 28th period. A similar survey of the graph of *O* shows that endomixis took place at periods 4, 9, 15 and 20. Subculture *O* died at period 25 from causes unknown. It was restarted from the main culture of this race at once and had endomixis at period 28. Subculture *B* was begun in period 18, that is immediately on the isolation of the race from 'wild' material. Endomixis occurred at periods 21 and 28.

Tabulation of these data shows that endomixis occurred in these subcultures as follows:

A at periods.....	4	9	15	21	28
AE " "	4 lost	9	15	0 died	28
O " "	4	9	15	20 died	28
B " "	–	–	—	21	28

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This experiment, in which different races were bred under the same varied culture conditions, shows an almost perfect synchronism of endomixis in all the races; that is, in *A* bred throughout without interruption on the varied medium; in *O* bred until its death near the end of the experiment on the same medium and again in the new *O* isolated from the main culture; in *B*, which was isolated from 'wild' material during the progress of the work; and finally in *AE*, which was restarted twice from a sister culture bred under "constant" environmental conditions.

The most reasonable conclusion, on the basis of this experiment, to account for the fact that the different races immediately showed endomixis synchronously, is that the general culture conditions initially influenced the appearance of the process; that is, brought about its consummation a few days earlier or later than it would have appeared under the former environment of the races, and that, once established, the rhythmic period characteristic of the species persisted and maintained the synchronism of endomixis. If this conclusion is justified then it must be assumed that the synchronism of *B* is due to the chance isolation of this race at just the same period in the rhythm which *A*, *AE* and *O* were at at the time, or so near this period that the environmental change immediately made it coincide with that of the other races. The alternate hypothesis would be that there is in all races of this species a definitely established synchronism which holds under all normal environmental conditions. But such a theory would require more than one series of experiments to render its discussion profitable!

B.

In this set of experiments a study was made of the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred under the same practically constant culture conditions, as already described. The results are shown graphically in Fig. 2.

Subcultures *At* and *AEt* (both derived from the same race, I, about 1,000 generations previously) showed endomixis in preserved specimens at periods 4 and 9 and *AEt* also at period 15. Neither culture again underwent the process during a

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period of over 100 days before it died, though the characteristic 'rhythms' in the division rate are apparent during this time. It is possible that the process did occur at about periods 19 and 25 (cf. Fig. 2) and was overlooked, but I believe that this is highly improbable in view of the thoroughness of the search.

Subculture *Ot* underwent endomixis at periods 4 and 9 and then died without repeating endomixis at the next expected period (cf. Fig. 2).

Subculture *Bt*, as the graph shows, had endomixis at the 28th period, just before the experiment was concluded. From the character of the curve it would be expected at period 23, but it was not observed.

The endomictic periods of the four subcultures (3 races) of *Paramecium* in this experiment may be tabulated as follows:

At at periods.....	4	9	0	0 died
AEt " "	4	9	15	0 "
Ot " "	4	9	0	– "
Bt " "	–	–	–	28

These experiments show three points of considerable interest. In the first place they corroborate, for races bred under constant culture conditions, what was found in the same races when bred under varied culture conditions in the experiment already described. That is, *At*, *AEt* and *Ot* show a perfect synchronism of endomixis, and this is most reasonably explained by assuming that the general culture conditions, at least initially, influenced the appearance of endomixis and that, once established, the rhythmic period characteristic of the organism persisted. *Bt* affords no data for comparison with the other races of this set of cultures, as they had ceased to show endomixis before *Bt* was started.

A second point of importance is that the 'rhythms' in the division rate are to a certain extent independent of endomixis—that is of the definitive series of nuclear phenomena—because the rhythms persist for a while in the absence of the morphological changes. One may suggest that the rhythms in the culture are an expression of the physiological conditions antecedent to the definitive onset of the nuclear changes—in other

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words that the cell has undergone the preliminary stages of endomixis which ordinarily call forth the observable nuclear changes but that in the cases in hand the latter were never realized. This is equivalent to making the term endomixis coextensive with the term rhythm—the term rhythm denoting the physiological effect as indicated in the reproductive activity, while the term endomixis covers all the underlying physiological

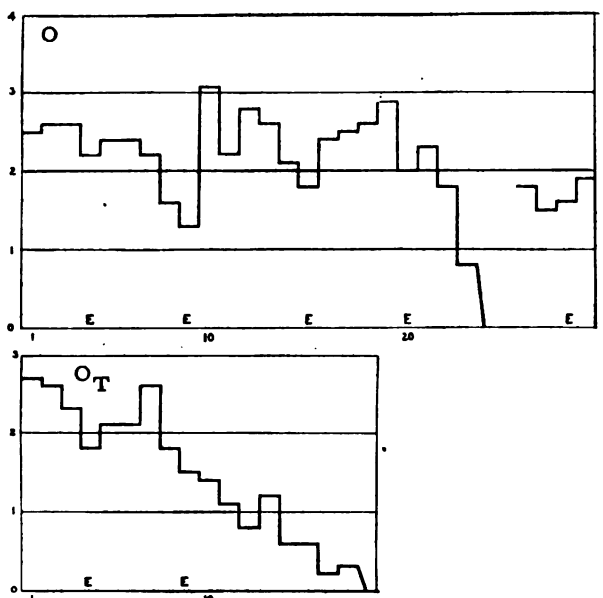

FIG. 5.

changes together with the definitive nuclear phenomena characteristic of the low point in the division rate. The term endomixis was *not* used in that broad sense when first employed by Woodruff and Erdmann and it is unprofitable to extend it now. Applying the term strictly to the complicated reorganization process of the cell does not deny the undoubted fact that these changes are but the expression of the climax of a series of physiological phenomena which probably extend back to the consummation of the previous endomictic period. So, it seems to be a more or less academic question whether rhythms and endomixis are independent. Certainly the rhythms occur for a while without endomixis in *sensu stricto*—but in all such cases the culture has died before very long.

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This leads to the third point of interest which is the death of the cultures after the cessation of endomictic phenomena. This is true in each instance: *At*, *AEt*, and *Ot* (cf. Fig. 2). The data from these experiments perhaps are not sufficiently extensive to make sure that this is not a case of *post hoc sed non propter hoc*, but they make it highly probable that endomixis is necessary for the continued life of the race.

C.

The data which have been presented in the study of the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred under varied and under constant culture conditions, may now be analyzed from the point of view of the periodicity of these phenomena in the *same* races under varied and under constant culture conditions, since experiments *A* and *B* of this series were conducted simultaneously.

This analysis is readily made by a study of Figs. 3, 4, 5 and 6, which consist of a combination of the graphs already presented from the other point of view. The graphs are paired, one above the other, so that identical five-day periods coincide. For example, in Fig. 3, period 10 of *A* is directly above the same period of *At*. Since the endomictic periods of all these cultures have just been considered, it is only necessary to tabulate them for reference:

A	underwent	endomixis	at	periods....	4	9	15	21	28
At	"	"	"	"	4	9	0	0	0 died
AE	"	"	"	"	4	9	15	0 died	28
AEt	"	"	"	"	4	9	15	0	0 died
O	"	"	"	"	4	9	15	20 died	28
Ot	"	"	"	"	4	9	0	—	died
B	"	"	"	"				21	28
Bt	"	"	"	"				0	28

From this table it is apparent that the synchronism of endomixis is practically perfect in the *same* races when bred under *different* environmental conditions. The first two experiments showed that it was the same for *different* races when bred under the *same* environmental conditions whether varied or constant. Therefore this experiment corroborates and broadens the conclusions derived from the former ones and shows clearly

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that the periodicity of endomixis is largely independent of the character of the culture medium—the general environmental conditions—within the rather wide limits in which it has been varied in parts *A*, *B* and *C* of this series of experiments. At most the culture conditions initially influence the appearance of endomixis. In other words, the organism is set, so to speak, to undergo endomixis approximately once a month and this it does under any more or less favorable environmental conditions. A sudden change, however, of these conditions may bring about endomixis slightly earlier than it otherwise would have occurred but after this the usual rhythmic period of the species is maintained.

Experiments—Series II.

The experiments of this series may be outlined as follows:

A. Study of the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred in a relatively *large* amount of culture medium supplied fresh *daily*.

B. Study of the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred in a relatively *small* amount of culture medium changed on *alternate days*.

C. Study of the periodicity of rhythms and endomixis in the *same* races of *Paramecium aurelia* when bred in a relatively *large* amount of culture medium supplied fresh *daily*, and in a relatively *small* amount of culture medium changed on *alternate days*.

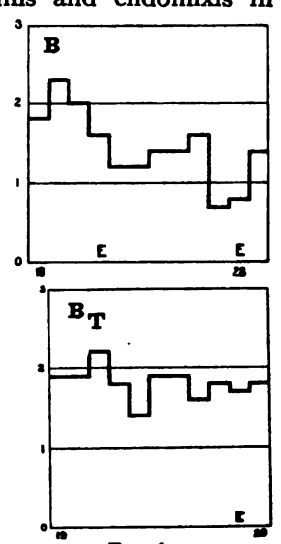

Fig. 6.

These experiments were begun on July 2, 1915, and continued until February 22, 1916. Five different races of the organism were employed. Three of them (*A*, *O*, and *B*) were the same subcultures which were used in the experiments of Series I., and which had been continued during the interim—that is, from March, 1915, to July, 1915. Therefore the numbering of the five-day periods was continued from the earlier work and the

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first period of the present series is number 53. Two new races were secured from diverse localities as already described and subcultures from these were begun and made a part of this experiment at once. These subcultures were designated *M* and *W*.

All these subcultures were supplied daily with the regular varied culture medium. Sister subcultures (designated *As*, *Os*, *Bs* and *Ms*) were isolated line by line from the above-mentioned subcultures at the start of the work and were subjected to the same varied culture medium but the amount was reduced to one half that supplied to the parent subcultures and the medium was changed at intervals of 48 hours instead of 24 hours.

It has been shown in previous papers,[1] that the rate of reproduction of *Paramecium* is markedly influenced by the volume and the freshness of the culture medium. This was found to result from the accumulation of the excretion products, in view of the fact that a medium which contains the excretion products of a heavy growth of paramecia has a decidedly depressing effect on the division rate of this organism.

Accordingly it seemed that the excretion products of *Paramecium* afforded the most natural means of quickly modifying the division rate in order to determine the effect of this on the rhythms and endomictic periods. Obviously the experimental conditions involve two variables—excretion products, and their effect, the lowering of the fission rate—so that the specific influence of one or the other cannot be determined from the data given below. But that is not of importance in the present work which is merely an endeavor, as already stated, to determine the effect of normal environmental changes.

A.

This set of cultures was carried on in order to study the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred in a relatively *large* amount of varied culture medium supplied fresh *daily*. The experi-

[1] Woodruff, "The Effect of Excretion Products of *Paramecium* on its Rate of Reproduction," *Journ. Exper. Zoöl.*, X., 1911; Woodruff, "The Effect of Excretion Products of Infusoria on the Same and on Different Species, with Special Reference to the Protozoan Sequence in Infusions," *Journ. Exper. Zoöl.*, XIV., 1913.

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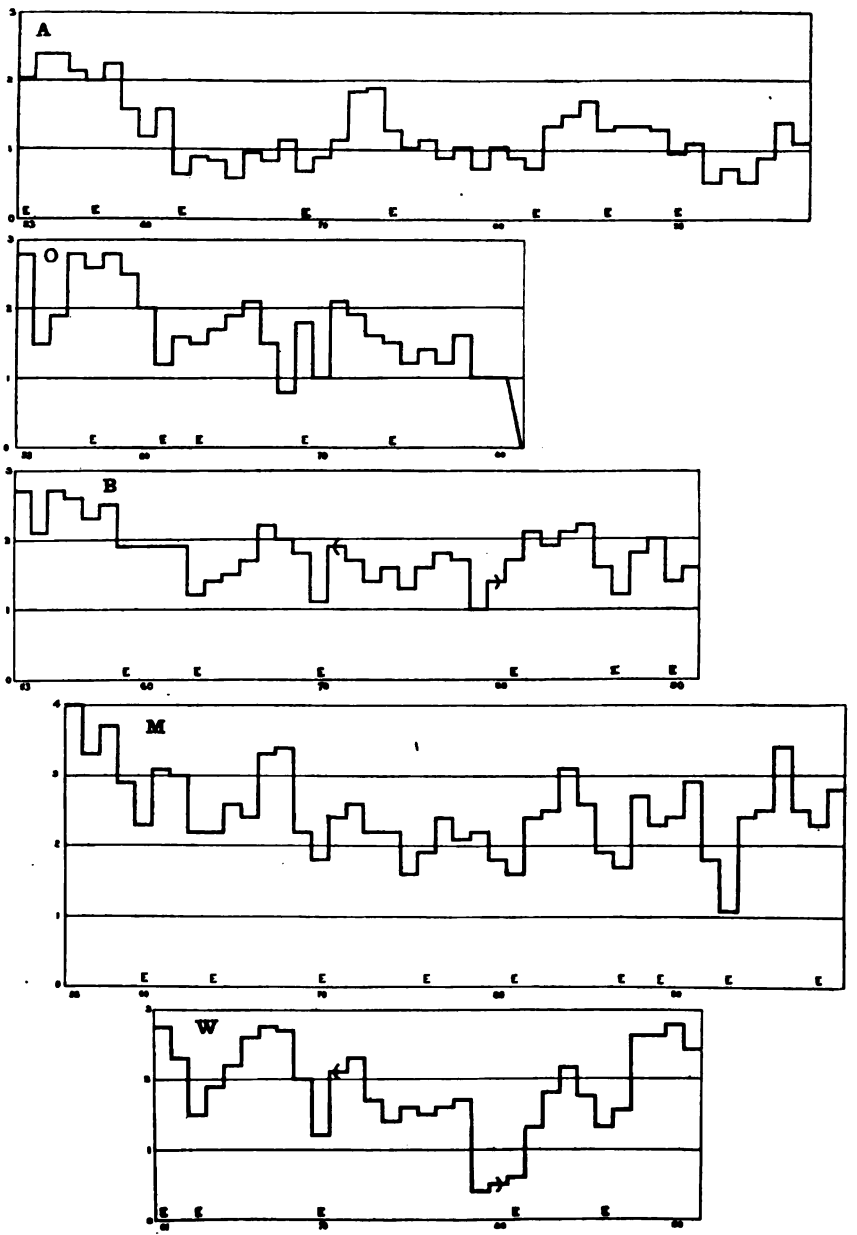

FIG. 7.

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ment is essentially a repetition of experiment *A* of Series I (cf. p. 443) since three races (*A*, *O* and *B*) and the character and amount of culture medium are the same as employed before. The results, therefore, now will be analyzed from the viewpoint of Series I. and later from that of the present series.

The results are shown graphically in Fig. 7 from which it is evident that:

A underwent endomixis at periods . . .	53	57	62	69	74	82	86	90		
O " " " " . . .		57	61–63	69	74					
B " " " " . . .		59	63	70	[1]	81	87	90		
M " " " " . . .		60	64	70	76	81	87	89	93	98
W " " " " . . .			61–63	70	[1]	81	86			

A study of this table and the graphs which it summarizes shows again practically the same periodicity of endomixis in diverse races on the varied culture medium as was observed in experiment *A* of Series I. The synchronism is not quite as exact as in the former experiment but, considering all the unknown and uncontrollable variables in such a long experiment, it clearly offers further support for the conclusion that rhythms and endomixis are essentially independent of environmental conditions, and that the culture conditions merely influence initially, if at all, the appearance of endomixis, and that once established the rhythmic period characteristic of the species is maintained within rather narrow limits.

B.

This set of experiments involves a study of the periodicity of rhythms and endomixis in *different* races of *Paramecium aurelia* when bred in a relatively *small* amount of culture medium changed on *alternate days*. The cultures used, as already stated, were *As*, *Os*, *Bs* and *Ms* and their behavior with respect to the process under consideration is given in Fig. 8. A study of this graph shows that endomixis was observed in:

As at periods	59	61	65	70	[1]	0	85	90
Os " "	59	0	64	69	[1]	82	86	
Bs " "	59	0	64	70	died			
Ms " "	59	62	64	70	[1]	82	87	91

[1] The animals were not studied cytologically during this period.

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Again the synchronism is very marked throughout the work, while during the initial period (59) it is perfect. The explanation of the occurrence of endomixis in every culture during the 59th period is undoubtedly due to the *marked* change of culture conditions to which these subcultures were subjected, when the experiment was initiated in period 58, by isolation of the 's'

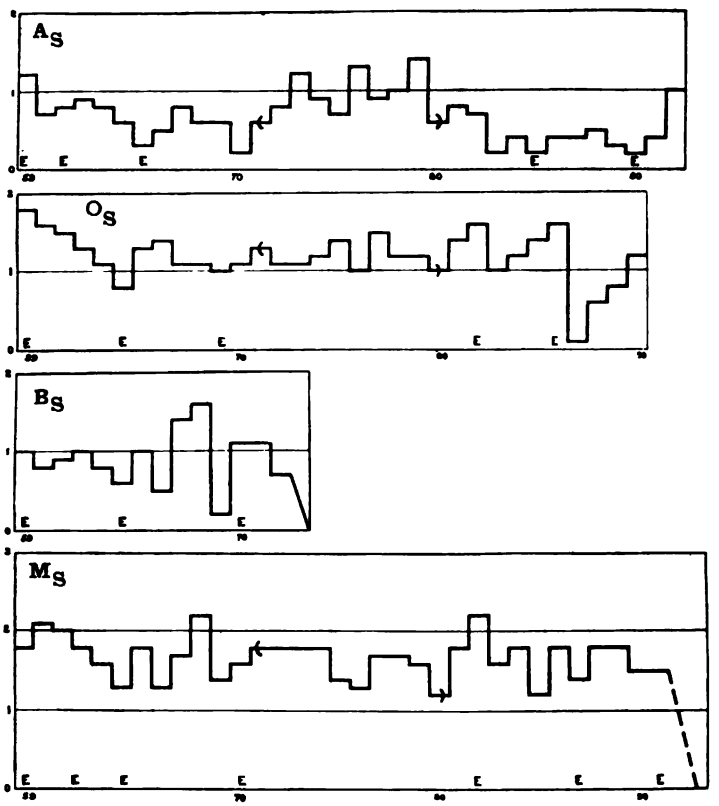

FIG. 8.

subcultures from their respective main cultures on the varied culture medium changed daily (cf. Figs. 9 to 12). This will be considered in connection with the following experiment, but obviously it is in accord with the results obtained by Woodruff and Erdmann[1] who noted in studying various *lines* of one race that the reorganization process "may occur a few days earlier in

[1] *Journal of Experimental Zoölogy*, 1914.

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animals not supplied daily with fresh culture fluid than in the regular lines."

It is apparent then from this experiment that the same general conclusion which was derived from all the previous ones is again justified, viz., the culture conditions may, at most, initially influence the appearance of endomixis; but once established the rhythmic period characteristic of the species is maintained with great exactness, resulting in a remarkable synchronism of the process in the different races. General normal environmental changes obviously do not permanently alter the fundamental inherent rhythmic periods of the organism.

C.

A study now can be made of the phenomena under consideration in the *same* races of *Paramecium aurelia* when bred in a relatively large amount of culture medium supplied fresh daily, and in a relatively small amount of culture medium changed on alternate days. This involves, obviously, the comparison of the results from the two previous experiments since these were conducted simultaneously and afford the requisite data. Therefore the culture graphs of these two experiments are presented, one above the other, so that identical five-day periods coincide, in Figs. 9, 10, 11 and 12.

It is to be noted that *As* and *Os* were branched from *A* and *O* very soon after endomixis had occurred in the latter cultures; *Bs* came from *B* during the actual occurrence of endomixis; while *Ms* was branched from *M* toward the end of a rhythm as the subsequent appearance of the process in *M* at period 60 shows.

The four figures mentioned and the following tabulation of the periods in which endomixis occurred in the various subcultures shows the synchronism of the reorganization process in all the pairs of cultures under the markedly different environmental conditions. It is difficult to say whether this coincidence of the process is more exact between different races under the same culture conditions or between the same races under different culture conditions, because most of the variations are so small that they fall well within the limits of error involved in the five-day plotting method, etc. (cf. p. 440).

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A at periods..	53	57	62	?	69	74	82	86	90
As " " ..		59	61	65	70	[1]	0	85	90
O " " ..		57	61	63	69	74	died		
Os " " ..		59	0	64	69	[1]	82	86	
B " " ..		59	0	63	70	[1]	81	87	90
Bs " " ..		59	0	64	70	died			
M " " ..			60	64	70	76	81	87	89
Ms " " ..		59	62	64	70	[1]	82	87	91

Thus clearly, in the long run, the *s* environment had no effect on the periodicity of the process. But, as pointed out before, endomixis appeared without exception in the four *s* cultures immediately upon their isolation from the respective parent cultures and on subjection to the stale culture fluid. Therefore, now that it is possible to compare parent and daughter lines, the obvious conclusion from the data is that endomixis was brought about earlier (except in *Bs* which was started during endomixis) by the changed cultural conditions; that is, earlier

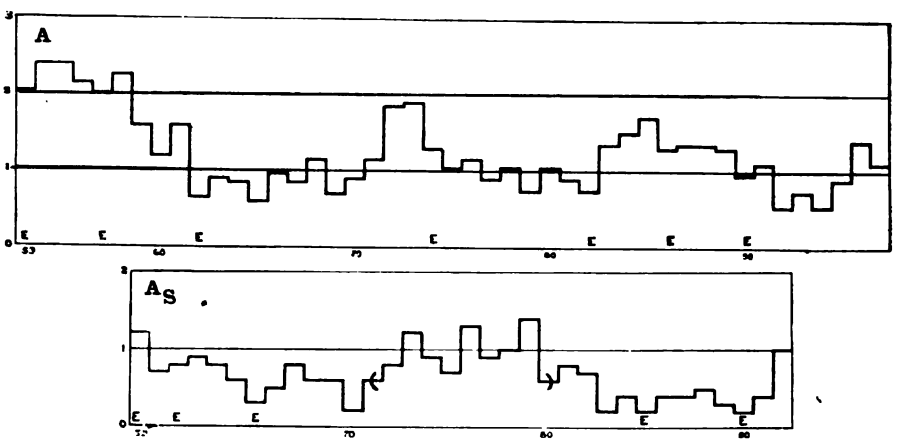

FIG. 9.

than it appeared in the parent cultures. This result, it may be noted, substantiates the conclusion from the former experiments that the remarkably exact synchronism of endomixis of various races under the most diverse environmental conditions is due to a slight initial influence on the occurrence of the process.

Figs. 9, 10, 11 and 12 show that the treatment to which the *s* cultures were subjected resulted in a distinctly lower rate of

[1] Not studied cytologically during this period.

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division—on the average about three quarters of division per day lower than in the cultures subjected to the fresh culture medium, etc. This is without doubt due, as already discussed, in large part at least to the accumulated excretion products in the *s* series. But whatever the cause, the experiment affords an opportunity to study the effect of naturally changed conditions, involving a lowered fission rate, on the periodicity of rhythms and endomixis.

Now since the *s* subcultures divided at a much lower rate than the parent cultures, and since endomixis appeared fairly synchronously in parent and *s* sets, it is obvious that endomixis consistently appeared in the *s* subcultures within a smaller number of generations. In other words, the treatment of the *s*

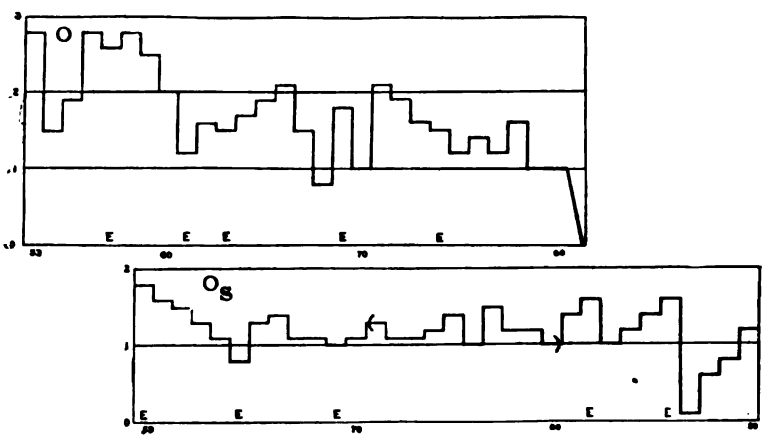

FIG. 10.

series apparently resulted merely in reducing the number of cell divisions in a given time and had practically no effect (except in the first period) on the occurrence of endomixis. On the basis of this set of cultures, then, endomixis is to a certain extent independent of the number of generations and more closely related to a time factor, if such an expression may be employed.

The *B* pair of cultures affords a fairly typical example (Fig. 11). In *B* endomixis occurred at periods 59, 63 and 70; while in *Bs* it occurred at periods 59, 64 and 70. Thus the length of

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time in days between successive reorganizations is 20 and 35 in *B*, and 25 and 30 in *Bs*. Therefore in both cultures the same number of days (55) elapsed from one endomixis to its second following occurrence. On the other hand in *B* the process was in progress at the 335th, 370th and 435th generations, that is at intervals of 35 and 65 generations, while in *Bs* it took place at the 335th (when *Bs* was isolated from *B*), 360th and 380th genera-

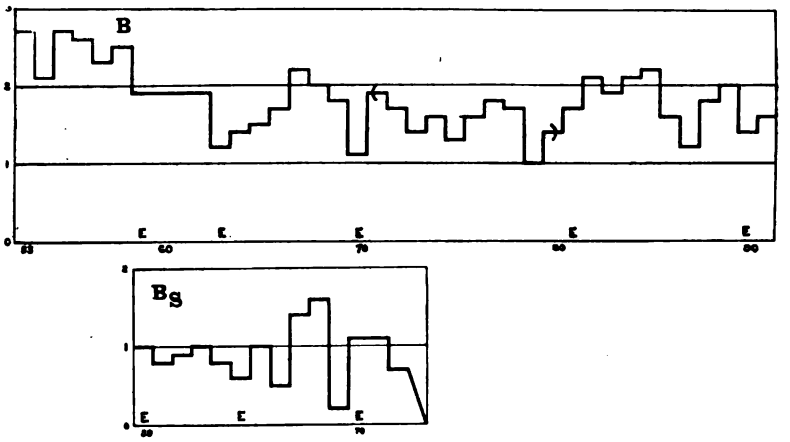

FIG. 11.

tions, or at intervals of 25 and 20 generations. Therefore in *B* 100 generations were attained from one endomictic period to the second following occurrence of the process; while in *Bs* only 45 generations occurred during the same period. To repeat: the experimental treatment apparently simply reduced the number of generations during the 100 days.

This same general result was obtained in Set *C* of Series I., though of course here the lowering of the division rate was the result of other causes. Figs. 3 and 4 show that endomixis occurred in the fourth period of the four subcultures, *A* and *At* and *AE* and *AEt*, although the rate of division and therefore the number of generations was less in *At* than in *A*, and less in *AEt* than in *AE*.

This set of experiments, then, corroborates in a clear-cut manner the general result derived from all the previous ones; that is, general environmental changes, especially if they are

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pronounced, usually bring on endomixis slightly earlier than it would have occurred if the cells had been left in the environment in which they were at the last reorganization period. But after this initial change the periodicity characteristic of the organism is resumed and persists.

In addition, however, this experiment suggests another point of interest: the length of the rhythm is apparently partially

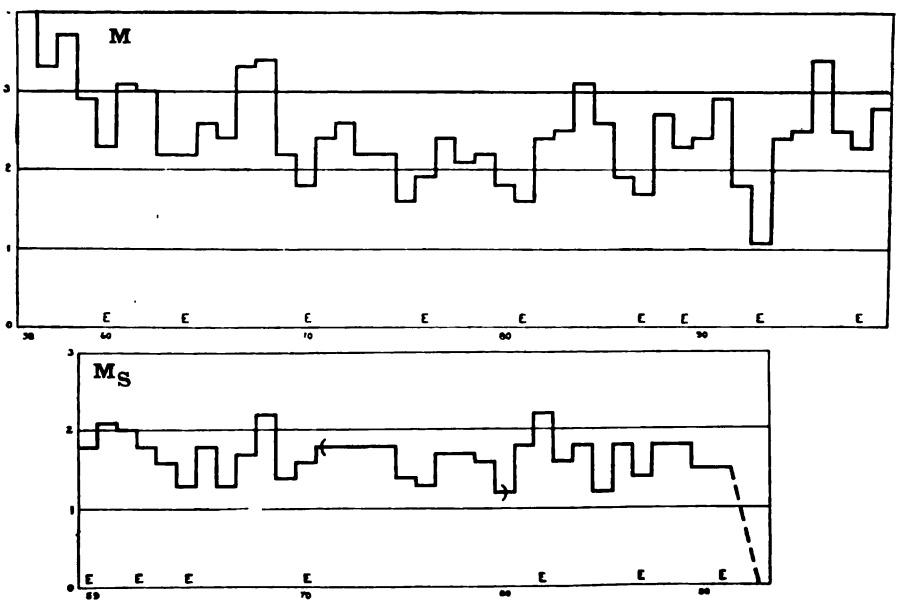

FIG. 12.

independent of the number of cell divisions—the periodicity being one in which time, so to speak, is an important factor. This is a most surprising result, because such profound reorganization phenomena as are involved in endomixis must bear a more or less definite relation to the physiological activity of the protoplasm, the best criterion of which is generally considered to be growth and reproduction as indicated by the division rate. More experiments obviously are needed to resolve this 'time factor' into its significant elements.

EXPERIMENTS—SERIES III.

Peebles states that Horlick's Malted Milk is a most satisfactory culture medium for *Paramecium* if used in a .2 per cent.

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solution,[1] and therefore this seemed to offer an opportunity to study the effect of a medium radically different in composition from those previously employed in these studies. Accordingly subcultures designated *Am*1, *Am*2, *Am*3, *AEm*1, *AEm*2, *Om* and *Mm* were started from *A*, *AE*, *O* and *M* respectively, and bred on this medium. The extent of these cultures and the time of appearance of endomixis (*E*) in the two sets is shown in the following table:

Period	52	53	54	55	56	57	58	59	60
A		*E*				*E*			
*Am*1	(from *A*)	*E*				*E*			
*Am*2	(" *A*)	*E*				*E*			
*Am*3						(from *A*)	*E*		
AE					*E*			*E*	
*AEm*1	(from *AE*)	*E*			*E*		discontinued		
*AEm*2						(from *AE*)	*E*		
O						*E*			
Om	(from *O*)					*E*			
M									*E*
Mm						(from *M*)	*E*		

This table shows that the malted milk medium did not change at all the periodicity of endomixis in either the *A* or *O* milk subcultures, while it consistently brought it about earlier in the *AE* and *M* milk series. The results from the *AE* and *M* cultures are therefore in accord with the results obtained in Series II of these experiments, while those with *A* and *O* apparently differ. However, the non-appearance of endomixis earlier in the *Am* series is readily explained by the fact that the process was just about to occur in the parent culture when the *Am* subcultures were branched, as is shown by the fact that it appeared in *A* during the following period.

The experiments with malted milk are obviously not extensive enough to give any details of the effect of long subjection to this medium on endomictic periodicity, but they adequately answer, it is believed, the purpose of the present study by again indicating that the appearance of endomixis may be slightly

[1] Peebles, "Regeneration and Regulation in *Paramecium caudatum*," BIOL. BULL., 1912.

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altered temporarily by subjecting *Paramecium* to a markedly changed environment.

Experiments—Series IV.

This series is a brief repetition of Series I., since it comprises a comparison of the periodicity of endomixis both in the same races when bred under varied and constant culture conditions, and in different races bred under varied and constant culture conditions. The cultures employed were *A*, *AE* and *B* which were used in the first series, and also two other cultures, *M* and *W*.

The following table shows a practically perfect synchronism of endomixis in all the races under the different environmental conditions:

Period	81	82	83	86	87	88	89	90
A		*E*		*E*				*E*
At2			(from *A*)	*E*				*E*
AE		*E*		*E*				*E*
AEt2			(from *AE*)	*E*				*E*
B	*E*				*E*			*E*
Bt2			(from *B*)	*E*				*E*
M	*E*				*E*		*E*	
Mt2			(from *M*)	*E*				*E*
W	*E*			*E*				
Wt2			(from *W*)	*E*				

This is exactly the same result which was obtained in the experiments of Series I. In Series I this synchronism was most satisfactorily accounted for by assuming that there was an initial effect on the periodicity by the change of environmental conditions to which all the cultures were subjected at the start. That this assumption was justified is shown by experiment *C*, Series II (cf. p. 455). However, it is to be noted in the present experiment that there is no apparent initial influence of the changed culture conditions, but this is probably due to the fact that the *A*, *AE*, *B*, *M* and *W* cultures had been so long under the same environmental conditions before their respective *t2* subcultures were branched from them. Consequently the syn-

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chronism of the *t2* set is due merely to maintaining the periodicity of the respective parent cultures.

General Summary.

All four series of experiments show that the general 'time-periodicity' of rhythms and endomixis in *Paramecium aurelia* is the same in the several races which have been studied under the following environmental conditions:

1. Varied culture medium changed daily, and at room temperatures.
2. Varied culture medium changed on alternate days, and at room temperatures.
3. Constant beef extract culture medium and at a temperature of 26° C.
4. Horlick's malted milk medium, and at room temperatures.

Thus it seems clear that one question which this study was planned to elucidate has been answered: General changes in the environment of the animals, as markedly different culture media and temperatures, such as may be termed normal changes, do not permanently modify the length of the rhythm or the time between successive endomictic periods which is characteristic of the species.

However, sudden marked changes in normal culture conditions may initially induce the appearance of the definitive endomictic phenomena slightly earlier than they would have occurred if the cell had been continued under its former environmental conditions; but this initial disturbance is soon compensated for, usually within the present rhythm, so that the previous characteristic periodicity is again resumed.

Throughout all the work there is evident a remarkable *synchronism* of the endomictic process in all the races bred simultaneously, regardless of the environmental conditions. Thus not only is the periodicity of endomixis, or length of the rhythm, the same, as stated above, but also the rhythmic periods are synchronous. The explanation of this is clearly due, in the experiments involving the most marked changes in the cultural conditions, to an initial effect of these changes, which brings into line, so to speak, the appearance of endomixis in all the cultures.

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Consequently it is highly probable that a slight initial shift of the definitive onset of endomixis in the various races is the explanation of the nearly simultaneous appearance of the process in all the races under all the conditions.

Although the 'time-periodicity' characteristic of the species has been shown by the present experiments to be practically unmodifiable under the general environmental changes which were employed, it has been found that the 'generation-periodicity'—or the number of cell divisions between one occurrence of endomixis and the next—may be modified to a considerable degree by the culture conditions which lower the division rate. In other words, the rhythm appears to be more susceptible of modification in regard to generations than time. As has been previously noted, this is a surprising result, since a profound reorganization process such as endomixis must be closely related to the general metabolism of the cell and this is expressed to a large extent in growth and reproduction. Further work on this problem is in progress.

Finally, the cessation of endomixis in these experiments was invariably followed, usually within a rhythm or two, by the death of the culture involved. This indicates strongly, if it does not prove, that a periodic occurrence of the definitive endomictic phenomena is a *sine qua non* for the continued life of the race—a conclusion which is concordant with all previous data in regard to endomixis.

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BIOLOGICAL BULLETIN

AUTOTOMY OF THE TAIL IN RODENTS.

F. B. SUMNER AND H. H. COLLINS.

It is perhaps not generally known that certain species of small rodents detach portions of the tail in a seemingly voluntary manner when they are seized by this member. Among the mouse-like rodents of California such an autotomy of the tail appears to be most typically exhibited by the pocket-mice (*Perognathus*).

It is the general practice of the writers, as probably of most breeders of mice, to pick up their animals by the tail. This appendage constitutes a convenient handle by which a mouse may be lifted, commonly relieving one of the risk of being bitten. But such a procedure is hazardous in the case of *Perognathus*. By a sudden gyratory movement of the body, the tail is likely to be severed at some point in its length, allowing the animal to make its escape and giving the pursuer something of a handicap in the race.

The whole performance is so suggestive of what occurs in many lizards that the question naturally arose in our minds: Can it be possible that autotomy, in the one case, as in the other, is followed by regeneration? This of course was not to be expected in a mammal, but the experiment seemed at least worth trying. In any case, the act and its consequences were thought to be worthy of a brief consideration.

The species upon which most of these observations were conducted was a rather large pocket-mouse, *Perognathus fallax fallax* Merriam, which is very common in the neighborhood of La Jolla. Despite the frequency of autotomy in this species, we soon found that all individuals cannot be depended upon to "perform" when desired. Those, in particular, which have been kept captive for some time seem to lose the tendency. More-

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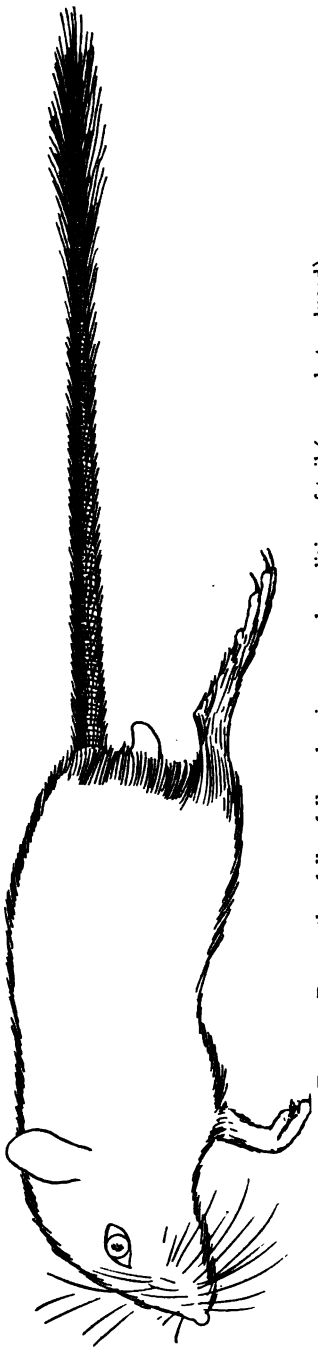

FIG. 1. *Perognathus fallax fallax*, showing normal condition of tail (somewhat reduced).

over, the act, when it does occur, is so sudden that one is commonly left wondering how it happened. But it was watched rather carefully in a number of instances, in order that the movements might be noted. In some cases, the body seemed to undergo a rapid whirling motion, comparable with that of a rope tied at one end to a post and held by the hand at the other. The snout and tail, in this comparison, correspond to the points of attachment, the hind quarters of the body to the whirling central portion of the rope. A number of individuals which did not actually detach the tail were found to undergo less violent gyrations of the same sort. In other instances, however, there seemed to be an actual torsion of the appendage, resulting from a rotation of the body. In yet another, where the tail was held down upon a cement floor, the detachment was effected by a sudden leap.

In eight cases, in which the detached portions of the appendage were carefully examined, it was found that the fracture invariably occurred across one of the caudal vertebræ, commonly near its center. The tail is likely to be broken at almost any point in its length. The detached portion carries away with it a group of tendons of such length as to indicate that these have their points of origin within the body

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itself. The possibility of a regeneration of the missing part of the tail was tested in fourteen specimens. Some of these underwent a voluntary autotomy, in the manner above described. In others, the breaking of the appendage was facilitated by the observer. These mice were all nearly mature, though probably most of them had not reached the limit of growth.

In the first seven specimens, the stump of the tail was measured shortly after the operation, but the length of the body was unfortunately not taken. Four of these specimens were living at the end of twelve months. In three cases, the tail has undergone a slight elongation (1–5 mm.), but this was doubtless merely an ordinary process of growth, accompanying the general growth of the body. In the fourth case, the recorded growth was 18 mm., though we believe this to be based upon an error in the first measurement. Except for the hair conditions, to be described below, there was no evidence of restoration of any part of the tail.

The next seven specimens were dealt with much more carefully. The length of the detached portion of the appendage was determined; likewise that of the stump, and the length of the body from the snout to the base of the tail.[1] Six of this second lot of mice were living at the end of nine months. When measured then, it was found that the tail stumps in five specimens had undergone a trivial increase in length, averaging about two millimeters. This was evidently incidental to a slight general growth of the body, since the mean body length, during the interval, had increased about four millimeters. In the sixth specimen, the tail was actually shorter, its condition indicating that it had been further damaged after the original operation.

Examination of the tissues of the tail in all of the foregoing specimens gave no suggestion of regenerative processes. In each case, the vertebral column terminated in a partial vertebra, this being evidently the one which was fractured in the process of autotomy.

There was, however, one very interesting result. In most of the specimens a dense tuft of elongated hairs had formed at the termination of the stump, the solid core of the latter being in

[1] In making the latter measurements the animal was etherized.

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some instances enlarged at the end. This tuft was entirely a new growth, since the middle region of the tail is normally covered with very short hair. As the uninjured tail of this rodent terminates in a "pencil" or tuft of longer hairs (Fig. 1) the formation of such a tuft at the point of amputation gave the appearance of a restorative process. There are, however, considerable points of difference between the normal "pencil" and the secondary one. The former is produced by the gradual elongation of the hair which covers the tail, commencing with the posterior third or half of this member. The restored tuft (Fig. 2) commences much more abruptly, sometimes being

FIG. 2. Tail which has undergone autotomy about midway in its length, and upon which a new terminal tuft of hair has been produced.

confined to the end of the stump. Its hairs are much longer than those of the natural pencil, and their pigmentation is conspicuously decreased, being pale gray on the normally black dorsal side.

In two instances a small lateral tuft was likewise formed, not far from the end of the stump. In one of these cases, there was found to be an internal injury, corresponding in position to this lateral tuft.

The capture of two wild specimens with truncated tails indicates that this accident may not be uncommon in nature. There was, of course, no clue to the source of the injury. Both specimens had developed terminal tufts, one of these being unusually perfect.

The time required for the formation of a new "pencil" has not been definitely noted. We have record of its having arisen within two months, but we also know that more than four months may be necessary.

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The writers offer no speculations as to the physico-chemical factors which bring about this excessive growth of the hair on the severed stump of the tail of *Perognathus*. A superficial analogy suggests itself between this terminal tuft and the cluster of adventitious shoots which arise from the stump of a tree. Possibly the analogy is more than superficial.

How general this process of autotomy is among the pocket-mice we can not say. We have noted its occurrence in only one other species, *P. panamintinus bangsi*, a small desert form in which the process is closely similar to that in *P. fallax*.

To what extent this phenomenon may be spoken of as a "protective adaptation," and whether or not it arose through natural selection, we are unwilling to conjecture. It is more than possible that a pursuing carnivore would sometimes be cheated of its prey in this manner, since the greatly elongated tail might readily be seized by the claws or teeth. The association between the fragile structure of this appendage and the peculiar instinctive responses of the animal is probably not accidental.

A phenomenon similar to that discussed above, but differing in essential features, has been observed by one of us in a species of *Peromyscus* (*P. boylei rowleyi*). This mouse likewise has a relatively long tail, and undergoes, when this member is seized, very much the same curious gyrations as does *Perognathus*. In the former species, however, the vertebræ are not broken, nor indeed is the central axis of the tail severed at all. The skin breaks at some point in its length, and slips off, leaving in one's hands the long tubular sheath which covered the appendage. This process is of such frequent occurrence as to be quite characteristic of these mice, which thus contrast strongly with the various other species of *Peromyscus* that we have observed. In the subspecies of *P. maniculatus*, for example, we have never observed the detachment of any portion of the tail or of the skin, in the course of handling several thousands of these animals. Nor have we observed it in any other genus of California Muridæ.

In *Peromyscus boylei rowleyi* we must again note what seems to be a significant correlation between structure and behavior. The skin of the tail breaks and slips off with remarkable facility.

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Likewise, the animal, when seized by this member, makes what appear like vigorous and well-directed efforts to accomplish the severance. In *P. maniculatus*, on the other hand, the skin is far less easily detached, and the animal has scarcely ever been observed to undergo either bodily gyrations or torsion of the tail.

To what extent this detachment of the skin is to be regarded as an "adaptive mechanism," which has arisen because of its utility, we regard it as futile to discuss at present. A single observation may be cited, which, however, can not be regarded as throwing much light on the matter. One specimen of *P. boylei rowleyi* was caught by the tail in a spring mouse-trap, the body being uninjured. Under these conditions, the animal was unable to escape by slipping off the skin and died of shock or exposure. On the other hand, it must be stated that a considerable proportion of the living mice of this species which were lifted by the tail underwent this mutilation, in spite of considerable care being taken to prevent it. What happens to the exposed portion of the tail after the detachment of the skin, has not been observed. It probably dies and falls off.

SCRIPPS INSTITUTION FOR BIOLOGICAL RESEARCH,
August 8, 1917.

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THE INTERRELATIONSHIP OF THE NUMBER OF STAMENS AND PISTILS IN THE FLOWERS OF *FICARIA*.

J. ARTHUR HARRIS.

I. Introductory Remarks.

A survey of the rapidly increasing literature must convince anyone that the problem of the factors which determine the sex of the organism is one of such complexity that it cannot be solved on the basis of any one kind of material or by any one method of research.

In the flowering plants the same individual may produce both eggs and sperm. The relative numbers of egg and sperm producing organs may vary from individual to individual, or from flower to flower within the individual.

It is reasonable to assume that definite genetic, morphogenetic or physiological factors underlie these variations. Any successful attempt to determine these factors and to measure their influence is just as truly a contribution to the wide problem of the physiology of sex as the more conventional breeding experiments and studies on the morphology of the germ cells.

The purpose of this paper is to point out certain hitherto unrecognized relationships between the number of sporophylls in the flower of the ranunculaceous genus *Ficaria*.

Heretofore those who have investigated the problem of the relationship between the number of stamens and pistils in the flower have been content to merely determine the correlation between the number of the two kinds of spore-bearing organs. Positive correlations of this kind should arise as the resultant of any sets of environmental factors which tend to increase both the number of stamens and the number of pistils in certain of the plants or individual flowers and to limit the number of both of these organs in others. Morphogenetically and physiologically it seems of far greater importance to inquire whether the relative

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proportion of the two types of spore bearing organs is correlated with the total number of sporophylls, which in lieu of any better character may serve as a measure of the total influence of intrinsic and extrinsic factors influencing degree of development.

Several years ago Professor Pearson and I (Harris, '09) showed that problems of this kind can be approached by determining the correlation between the total organs laid down and the deviation of the number of a particular kind from the probable number on the assumption that the proportion of the particular kind is independent of the total number.

The statistical method may of course be applied to experimental data or to series of determinations made on organisms developing under natural conditions. As yet experimental series are not available.

In a former paper ('16) I showed that in the inflorescences of both *Arisarum vulgare* and *A. proboscidium* there is a significant negative correlation between the total number of flowers and the deviation of the number of staminate flowers from their probable number on the theory of proportional distribution. Thus the male flowers while *absolutely* more numerous in the inflorescences with larger total numbers of flowers are *relatively* less numerous than in the inflorescences with smaller total numbers of flowers. Or, conversely, the larger inflorescences tend to produce *a larger proportion* of pistillate flowers.

In this paper the same analytical methods will be applied to the problem of the relationship of the number of stamens and the number of pistils to the total number of stamens and pistils produced by the flower.

II. Materials.

The materials upon which the coefficients discussed in this paper are based have been tabled and the chief biometric constants deduced by competent statisticians. The special methods upon which the conclusions of this paper are based were not, however, available at the time their calculations were made. The results are, therefore, quite new.

The series employed are the following:

1–2. A series of 283 countings of number of stamens and

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pistils of *Ficaria verna* from Trogen and another series of 80 countings from Gais, published by Ludwig ('01). Statistical constants for both of these series have been deduced and published by Dr. Alice Lee ('02).

3–4. A series of 268 early and 373 late flowers of *Ficaria ranunculoides* collected by MacLeod ('99) and discussed by W. F. R. Weldon ('01).

5–8. Four series of *Ficaria ranunculoides* collected by Galton, Weldon, Pearson ('03) and others in Italy, Guernsey and England.

III. Presentation of Data.

The means and variabilities of number of stamens and pistils per flower have been given in the papers cited. The only point which requires discussion in this place is the relative variability of the number of the two types of sporophylls. This is shown in Table I.

Table I.

Relative Variabilities in Number of Stamens and Number of Pistils in *Ficaria*.

Series.	Number of Flowers.	Coefficient of Variation for Pistils.	Coefficient of Variation for Stamens.	Differences
Switzerland—				
Trogen, I	283	23.32	18.68	4.64
Gais, II	80	23.73	12.18	11.55
Belgium—				
Early, III	268	23.32	14.07	9.25
Late, IV	373	27.89	18.46	9.43
Italy, V	624	22.35	14.12	8.23
Guernsey, VI	520	26.54	17.16	9.38
England—				
Dorset, VII	505	26.38	16.84	9.54
Surrey, VIII	500	27.19	17.32	9.87

The number of pistils is consistently more variable than the number of stamens.

Other workers have shown that there is a correlation of medium intensity between the number of stamens and the number of pistils per flower. Their constants, all of which have been rechecked in the course of this work, are shown in Table II. I have also added the linear regression equations showing the rate of increase in mean number of pistils associated with an increase in the number of stamens and the rate of increase in

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TABLE II.

CORRELATION BETWEEN NUMBER OF STAMENS AND PISTILS IN *Ficaria* AND REGRESSION EQUATIONS FOR STAMENS AND PISTILS.

Series.	Number of Flowers.	Correlation Stamens and Pistils.	Ratio of Correlation to Probable Error r/E_r.	Regression Line.
Switzerland—				
Trogen	283	.530 ± .029	18.27	$S = 11.708 + .645P$
				$P = 4.461 + .429S$
Gais	80	.388 ± .064	6.06	$S = 19.075 + .262P$
				$P = 4.403 + .575S$
Belgium—				
Early	268	.507 ± .031	16.35	$S = 18.197 + .489P$
				$P = 3.427 + .525S$
Late	373	.749 ± .015	49.93	$S = 9.006 + .729P$
				$P = -1.593 + .769S$
Italy	624	.439 ± .022	19.95	$S = 19.313 + .418P$
				$P = 5.409 + .460S$
Guernsey	520	.534 ± .021	25.43	$S = 18.302 + .404P$
				$P = 4.160 + .707S$
England—				
Dorset	505	.669 ± .017	39.35	$S = 20.369 + .535P$
				$P = -1.333 + .835S$
Surrey	500	.660 ± .017	38.82	$S = 19.091 + .588P$
				$P = -.860 + .741S$

mean number of stamens associated with an increase in number of pistils per flower.

The regression of the number of stamens on the number of pistils and of the number of pistils on the number of stamens is shown for three of the larger series in Figs. 1–3.

The empirical means for the Italian series, Diagram 1, do not conform very satisfactorily to the lines given by the equations. Better agreements between actual and theoretical means could hardly be found (in series of data no larger than these) than in the Guernsey and Surrey series represented in Figs. 2 and 3.

The main purpose of the present paper is to present the results of the determination of the relationship between the total number of sporophylls and the number of stamens and pistils.

The correlations between the total number of sporophylls and the number of stamens and pistils are shown in Table III.

As is to be expected the correlations between total sporophylls and number of stamens and pistils are high.

The constants showing the relationship between the total number of sporophylls and the deviation of the number of

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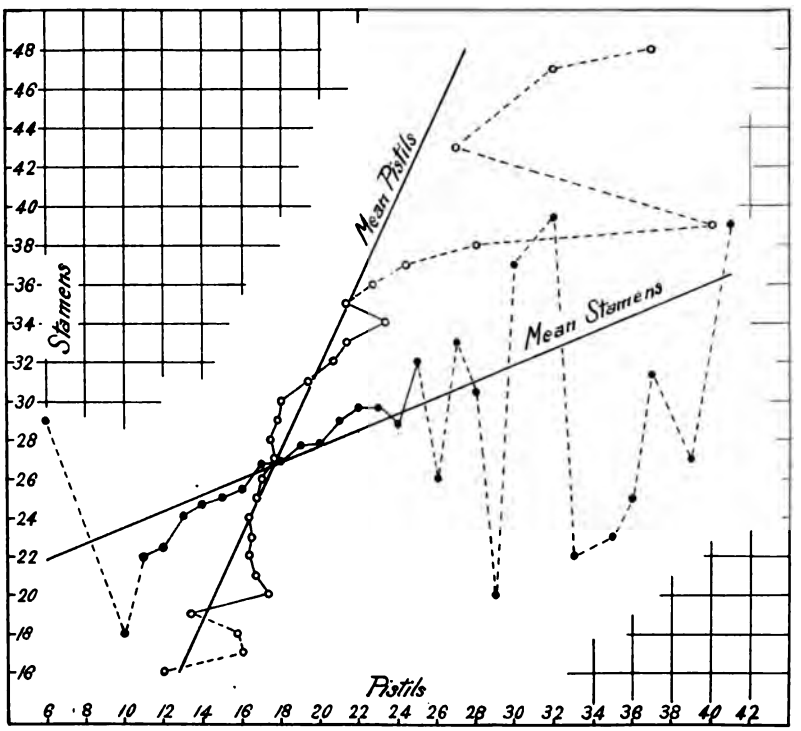

FIG. 1. Average numbers of stamens in flowers with various numbers of pistils and average numbers of pistils in flowers with various numbers of stamens. Empirical and smoothed values. Italian series.

TABLE III.

CORRELATION BETWEEN TOTAL SPOROPHYLLS AND NUMBER OF STAMENS AND PISTILS AND BETWEEN TOTAL SPOROPHYLLS AND DEVIATION OF THE NUMBER OF STAMENS AND PISTILS FROM THEIR PROBABLE VALUE.

Series.	Correlation Between Sporophylls and Stamens.	r_{ss_s}.[1]	r/E_r.	Correlation Between Sporophylls and Pistils.	r_{ss_p}.[2]
I.	.901 ± .008	− .139 ± .039	3.52	.845 ± .012	+.139 ± .039
II.	.755 ± .032	− .548 ± .053	10.37	.896 ± .015	+.548 ± .053
III.	.862 ± .011	− .378 ± .035	10.69	.873 ± .010	+.378 ± .035
IV.	.933 ± .005	− .477 ± .027	17.74	.936 ± .004	+.477 ± .027
V.	.840 ± .008	− .354 ± .024	14.99	.855 ± .007	+.354 ± .024
VI.	.836 ± .009	− .433 ± .024	18.04	.910 ± .005	+.433 ± .024
VII.	.892 ± .027	− .487 ± .023	21.37	.932 ± .004	+.487 ± .023
VIII.	.900 ± .006	− .463 ± .024	19.64	.921 ± .005	+.463 ± .024

[1] Correlation between sporophylls and deviation of stamens from their probable value.

[2] Correlation between sporophylls and deviation of pistils from their probable value.

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macro- and the number of microsporophylls from their probable value are the coefficients of critical value. These are also given in Table III. The correlations for stamens and pistils are necessarily equal in magnitude but opposite in sign. They show that the number of pistils is relatively larger in the flowers with larger numbers of sporophylls. The results are consistent in sign throughout. All of the correlations except that for the series from Trogen may be considered certainly significant in comparison with their probable errors.

While the constants for certain of the series differ significantly, the results are (considering the relatively small numbers and the very wide geographical distribution of the material) very consistent. Five of the eight series differ from $r = \pm .50$ by less than twice their probable error. Of the other three series, only Professor Ludwig's Trogen material is very aberrant.

For two of the series I have determined the standard devia-

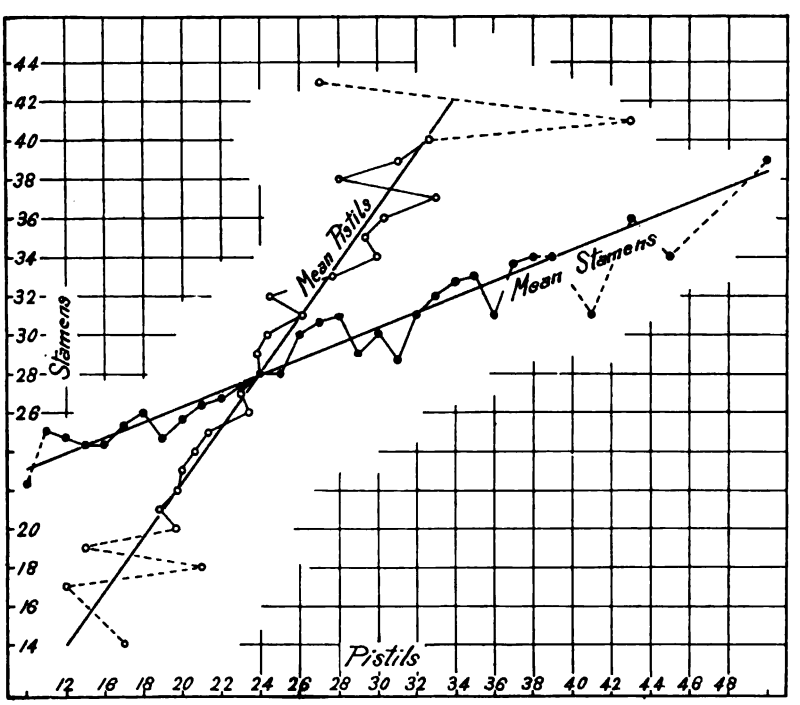

FIG. 2. Empirical means and regression straight lines for regression of stamens on pistils and pistils on stamens. Guernsey series.

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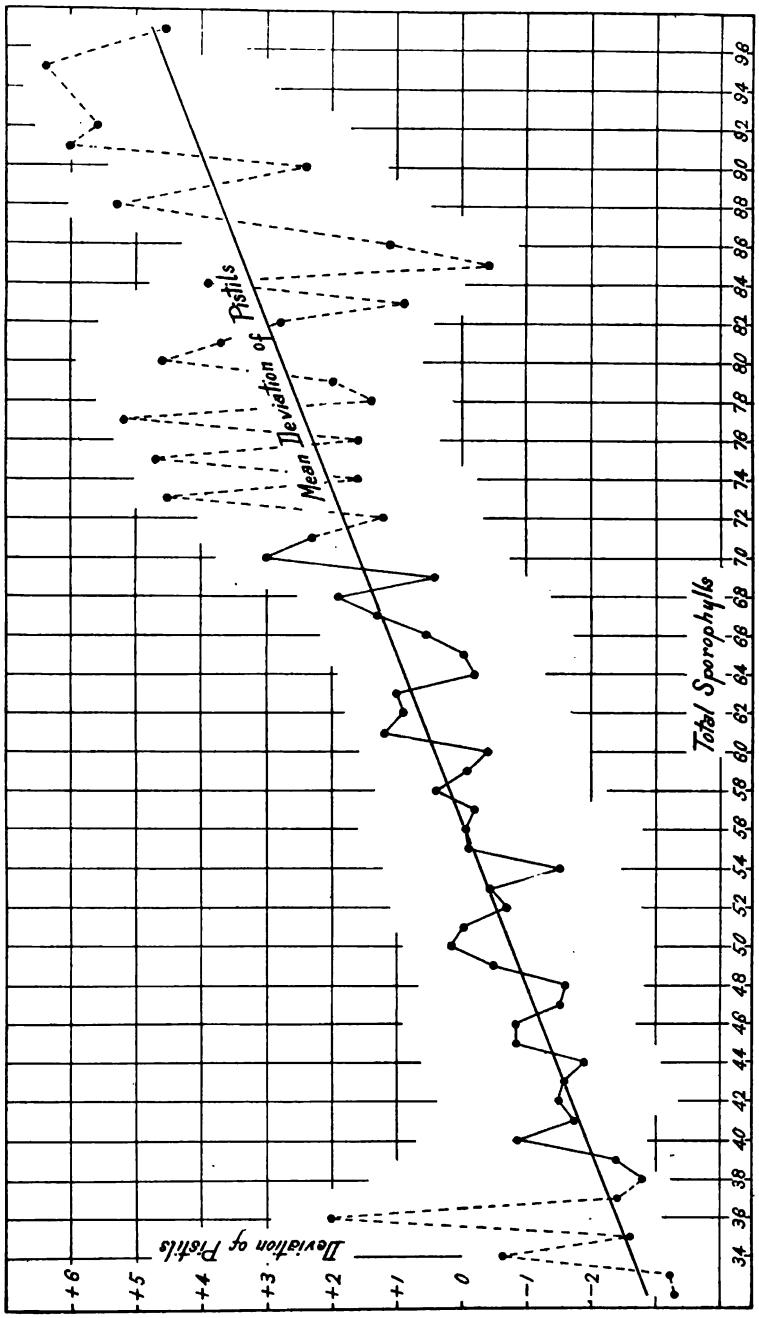

FIG. 5. Regression of the deviation of the total number of pistils from their probable value on the total number of sporophylls. Surrey material.

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IV. Recapitulation.

The present study deals with the problem of the relationship between the total number of sporophylls laid down and the relative number of stamens and pistils in the ranunculaceous genus *Ficaria*.

Constants have been deduced for eight series of published data from Italy, Switzerland, Belgium, Guernsey and England not hitherto analyzed by the methods now available.

In flowers with larger numbers of sporophylls the pistils are relatively more numerous than the stamens. The high degree of consistency of the results drawn from such a wide range of habitats indicates that the relationship is one of real morphogenetic significance.

In an earlier paper it has been shown that in *Arisarum* the relative number of pistillate flowers increases as the total number of flowers becomes larger.

It is at least suggestive in relation to the problem of the physiology of sex that in both of these very different forms the number of macrosporophylls becomes relatively larger as the total number of sporophylls increases. The relationships may, however, have an embryological explanation. Only further investigations will justify final conclusions concerning the cause of the relationship demonstrated.

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SOME OBSERVATIONS ON ARTEMIA GRACILIS, THE BRINE SHRIMP OF GREAT SALT LAKE.[1]

ALBERT C. JENSEN.

Very little investigation has been made of the animal life of Great Salt Lake. This is probably because in popular literature we still read that no living thing can exist in the brine of the lake, or because the few forms adapted to such life have failed to interest scientists. So far as the author knows, no attempt has been made to make a complete scientific classification of the lake fauna or to do any extensive research work on it. The brine shrimp, *Artemia gracilis*, one of the lake forms, is a small crustacean found in great abundance.

In referring to the literature on the subject, we find that Captain Bonneville wrote short descriptions of his explorations along the shores of Great Salt Lake, making mention of small animals in the water, probably the brine shrimp, as early as 1831–1833. Fremont also states of rowing to an island in the lake where he found what he called larvæ of insects washed upon the shore in great heaps or windrows three or four feet wide, and many animals living in the water. Verrill described the brine shrimp as *Artemia gracilis* in 1869. Seven years later Dr. Siebold, of Munich, obtained through Dr. Hagen, of Cambridge, Mass., a quantity of dried mud from Great Salt Lake which contained fertile eggs of *Artemia*. These eggs were hatched in artificially prepared brine and shrimps reared therefrom. Siebold succeeded in propagating *Artemia* for several generations and was quite convinced that this crustacean reproduces parthenogenetically as well as sexually. Packard ascribes the abundance of this phyllopod in Great Salt Lake to the absence of enemies. Further, after an examination of specimens he concluded that Verrill's later described species are merely different stages of the same species and should be included under the first described form, *Artemia gracilis*. As late as 1889 David Starr Jordan stated

[1] Contribution from the Zoölogical Laboratory of the University of Utah.

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that the lake water was so salty that no life could exist in it except the brine shrimp. Gilbert suggests that the presence of *Artemia* is no mystery for its ancestors may have lived in the fresh water of the basin and have transformed with the increasing saltiness of the lake. Talmage observed that the little phyllopod existed in great numbers in the lake, especially between the months of May and October. He states having collected them in the midst of winter when the temperature was far below the freezing point of fresh water, and from the evaporating ponds of the salt works, where the brine was near the point of saturation. He was able to keep them alive for several days in various dilutions of lakewater, and noted that they would even live for some time in distilled water. He further observed that repeated washings in fresh water for five minutes removed the brine so completely that salt had to be added to make them a palatable food.

The adult *Artemia*, Plate I., has a small head carrying a single black median eye (ocellus), a pair of large stalked compound eyes, two pairs of antennæ, one pair of mandibles and a pair of maxillæ. The second pair of antennæ of the female are short and pointed, while those of the male are large and broad, Plate II. The thorax bears eleven pairs of legs which are leaf-like in structure and serve as swimming organs and for the attachment of the respiratory organs. The abdomen is long, composed of eight segments without appendages. A number of bristles are to be found on the terminal segment. The ovisac is roughly bottle-shaped, being rather short and broad and attached to the first and second abdominal segments.

The œsophagus is short and leads to the stomach, which is situated in the head. The stomach is divided into two lobes, each of which receives a duct from the liver. The liver is two-lobed and lies in the head anterior to the stomach. The intestine is a straight tube. The division extending through the thorax is twice as large as that passing through the abdomen. The intestine includes a well-marked rectum, which is provided with constricting circular muscles. The ovaries are located in the first and second abdominal segments ventral to the intestine. The testes are located in the same relative position. The heart

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is a long tube beginning just posterior to the mandibles and terminating in the anterior part of the last abdominal segment. It has a distinct valve at the posterior end and apparently a pair of valves for each segment, Fig. 1. The blood enters the heart through these side ostea and is forced out through the ends of the heart. The central nervous system consists of a two-lobed supraœsophageal ganglion in front of the œsophagus, and just behind the œsophagus, between the digestive canal and the floor of the body, is the subœsophageal ganglion, the first of a chain that extends through the body along the ventral side. Nerve fibers extend from these ganglia to the body and appendages.

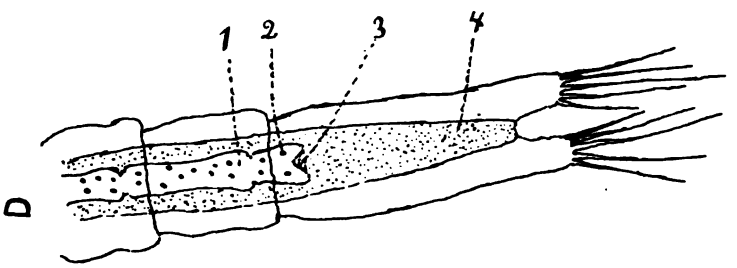

FIG. 1. Last three abdominal segments. 1, Heart; 2, blood corpuscles; 3, terminal valve; 4, intestine.

The sexes are separate in *Artemia*. The female is 10 to 12 mm. long, while the male has a length of 8 to 10 mm. Reproduction is accomplished by means of eggs that may or may not be fertilized.

In copulating the male claspers are placed around the body of the female just in front of the ovisac and thus attached the pair may swim about for several days, as many as five having been observed. At intervals the male's abdomen may be seen to bend so that the genital appendages come into contact with the oviduct. Frequently copulation takes place before the ovisac begins to show a brown color, due to the color of the eggs, and in such cases the eggs are retained in the ovisac for as long as three days after copulation has ceased. In other instances copulation does not take place until the eggs are brown in the ovisac and often these are deposited and even hatched while copulation is still in progress. It is presumed that such a copulation is too

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late to fertilize the eggs for they have passed from the ovaries into the ovisac and are covered with a brown shell and are ready to be deposited. These eggs, deposited during April and May, hatch within twenty-four hours after being laid.

A comparison of these newly deposited eggs with those collected during the early spring showed no particular difference, except that none of the new eggs were cup-shaped as are many of the winter eggs, Plate III., *A*. Examination of a number of females showed the number of eggs to vary between sixteen and one hundred and twenty. The females deposit their eggs while swimming.

Eggs were collected March 8 and kept in the laboratory in normal lake water for twenty-one days. The room temperature apparently had little effect upon the development since nauplii were collected in the lake three days after individuals were observed in the aquarium.

The embryo transforms into a nauplius much unlike the adult, Fig. *E*, Plate III. It has a blood-red color, a median eye (ocellus) and three pairs of appendages, the first of which develop into the first pair of antennæ, the second pair are much larger and serve as swimming organs, while the third pair become the mandibles. After a few days the second pair, in females, become shorter, less movable, lose their bristled margins, and are transformed into small scarcely movable processes, the second antennæ, Plate II., *B*. In the males they also form the second antennæ which develop into disproportionately large claspers with broad lobes, Plate II., *A*, and are used as catching and clasping organs. Along with the early development of these are signs of segmentation which is followed by the appearance of the thoracic appendages one pair after another until all eleven pairs have been laid down. The stalked compound eyes appear about the same time as the first thoracic appendages. Within eighteen to twenty-one days the animals reach sexual maturity, shown by copulation. Sexual differences become apparent when the animals are a little more than half grown. The males are stronger and more active than the females.

Siebold's conclusion that *Artemia* may reproduce parthenogenetically is verified by my observations. Water was taken

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from the lake before nauplii appeared there, and precautions taken to eliminate all eggs. Undeveloped females were placed in this water, where they developed normally and deposited eggs similar in appearance to fertilized ones. These eggs produced males and females, which developed normally and as far as could be observed could not be distinguished from those coming from fertilized eggs. A large proportion of the hatch was females. Both methods are evidently used during the summer months.

According to my records all of the brine shrimps die during the winter months, probably due to the lowered temperature. These observations do not harmonize with those of Talmage, who says: "I have taken them in the midst of winter, when the temperature of the water was far below the freezing point."

Statements made by Gilbert that the ancestors of *Artemia* may have lived in the fresh water of the basin, led to an experiment to determine the effect of different concentrations of salt water upon this animal. By using distilled water and the process of evaporation, solutions were prepared having a specific gravity as follows:

I.	II.	III.	IV.	V.	VI.	VII.	VIII	IX.
Dis. water					Normal lake water	Evaporated to	Evaporated to	Evap. to saturated point
1.000	1.027	1.044	1.064	1.085	1.109	1.130	1.711	1.179[1]

On March 28 eggs were put into the above solutions. March 30, 44 hours later, nauplii had emerged and were swimming about in solutions 1, 2 and 3 and in 4 they were emerging from the eggs. In the latter I observed the ocellus, also one or two pairs of appendages that were moving as if trying to free the body from the shell. By April 10 nauplii were emerging in solution 5 and not until April 13 did the eggs in solution 6 begin to hatch. By this date nauplii were also emerging from eggs in the lake. On May 5 a few nauplii appeared in solution 7, but in solutions 8 and 9 no nauplii had emerged. To test if the eggs in these last two solutions were fertile, a few were put into a less dense solution where they hatched readily. In solution 1

[1] The specific gravities herein given are the uncorrected hydrometer readings.

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eggs hatched readily in twenty-four hours, but the nauplii lived only from one to three days. However transferring nauplii from 1 to solutions 2 to 6 inclusive brought good results. In solutions 2 to 6 the nauplii, hatched in the order stated above, lived and developed rapidly to maturity. Young and adult *Artemia* transferred to solutions 8 and 9 from less dense solutions died within five to ten hours, the young dying first. Those put into solution 7 developed slowly, while those placed into solutions 2 to 6 developed quite normally. Solutions 3, 4 and 5 however seemed to be the most favorable for the growth of *Artemia*. They would also develop normally when transferred among any of the solutions 2 to 6 inclusive.

Artemia is often found in great numbers. Fremont in writing of his explorations of the lake speaks of windrows of the brine shrimp and the pupa cases of insects washed upon shore. Talmage states having seen the surface of the water tinted by them and also of having gathered with a tow-net a quart within a few minutes. Indians inhabiting this region used to collect large quantities of the crustacean along with insect larvæ which they dried and used as food.

The brine shrimp is a vegetable feeder. Near shore may be seen many masses of an alga, *Aphanothece*, and it is along with this that *Artemia* is most abundant. This alga and its gelatinous secretion forms the principal food for the phyllopods as shown by the fact that they may be seen feeding upon it, and it can also be demonstrated in their digestive tracts. In fact this alga seems to be their only food.

Artemia gracilis is chiefly a littoral form, since in this zone its food is most abundant. However these crustaceans have been observed by Talmage in great swarms far out in the lake where they were carried by winds unless they have swarming or migratory habits.

There is a very disagreeable odor encountered upon approaching the shores of Great Salt Lake at certain places. This stench has been attributed to the decomposition of the brine shrimp. The odor is at times very offensive, which, by some, is thought to be dangerous, and as a result the summer resorts of the lake are not visited by many people who would otherwise enjoy them.

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The stench is due primarily to decaying vegetable matter. Vessels placed in the laboratory containing plant material from the lake gave off the characteristic lake odor. Further the odor was present at the lake as early as the last of March, before the appearance of *Artemia*. Again the proportion of the phyllopods to the plant material is so small as to make the brine shrimp an insignificant factor in the odor production.

Conclusions.

Winter eggs of *Artemia* kept in normal lake water at room temperature (about 20 degrees centigrade) for a period of two months did not hatch earlier than those in the lake.

The embryo of *Artemia* develops into a free-swimming nauplius, much unlike the adult, which passes through a series of stages before reaching maturity.

Artemia reproduces by means of fertilized eggs and also parthenogenetically.

Eggs will hatch during the winter as well as spring and summer in dilute lake water or in fresh water at a temperature as low as 20 degrees centigrade. My experiments show also that eggs will not hatch in solutions concentrated to near the saturation point.

Artemia passes the winter months in the egg stage.

Artemia will not live in fresh water, but will in a dilution of the lake water having a specific gravity of 1.027.

Lake water diluted to a specific gravity of 1.044 to 1.027 is most favorable for the development of *Artemia*.

I take this opportunity to heartily thank Dr. Newton Miller for his assistance in this work.

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EXPLANATION OF PLATES.

All figures drawn with the camera lucida.

PLATE I.

Adult female *Artemia gracilis*.

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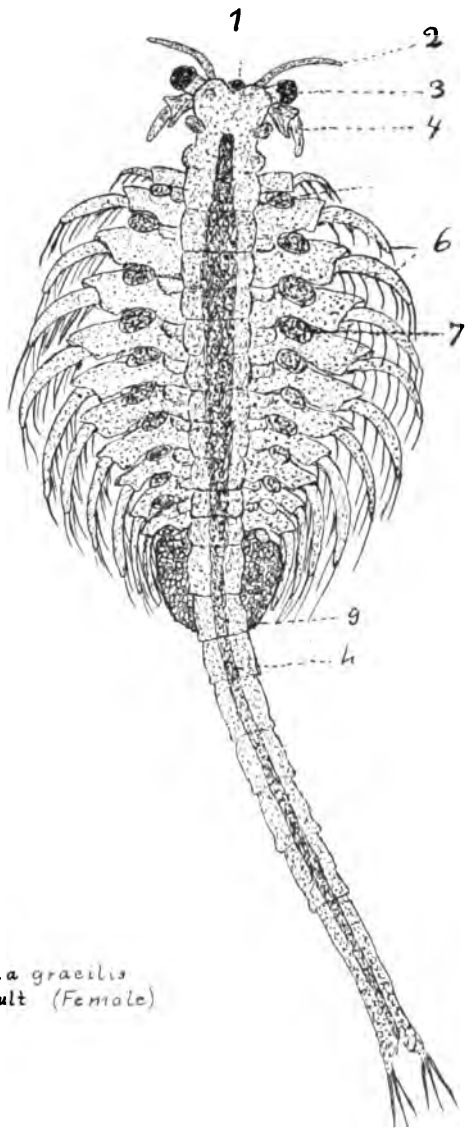

Artemia gracilis
Adult (Female)

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PLATE II.

A. Head of male showing first pair of antennæ.
B. Head of female showing first pair of antennæ.
C. Mandible.
D. Compound eye.
Figures of this plate drawn on the same scale.

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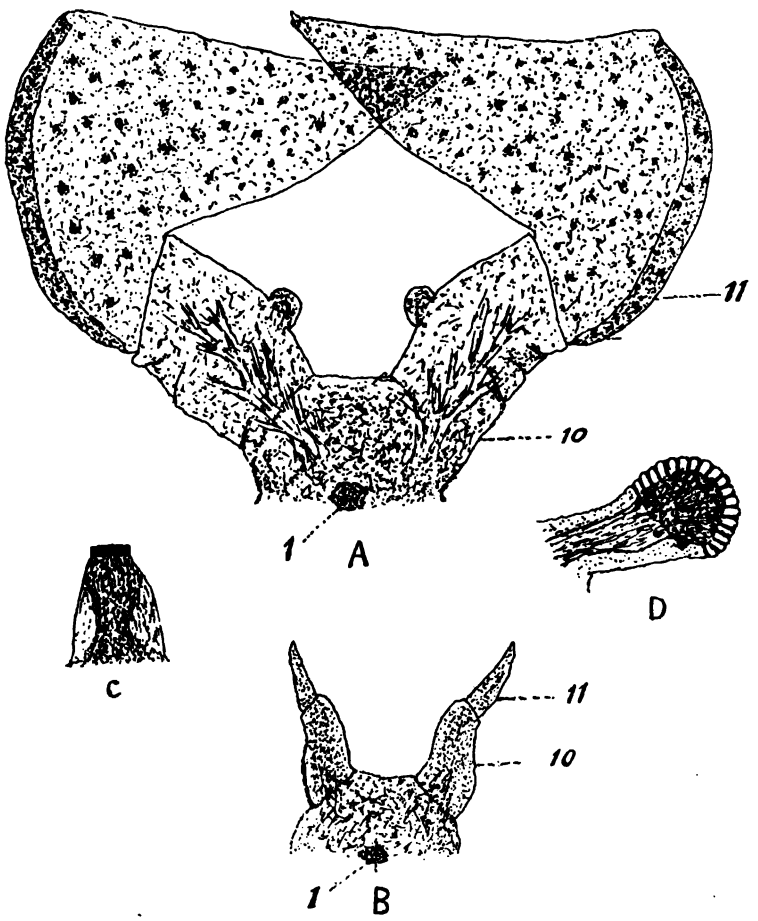

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PLATE III.

A. Cup-shaped egg.
B. A spherical egg.
C and *D*. Nauplii emerging from eggs.
E. Early nauplius stage.

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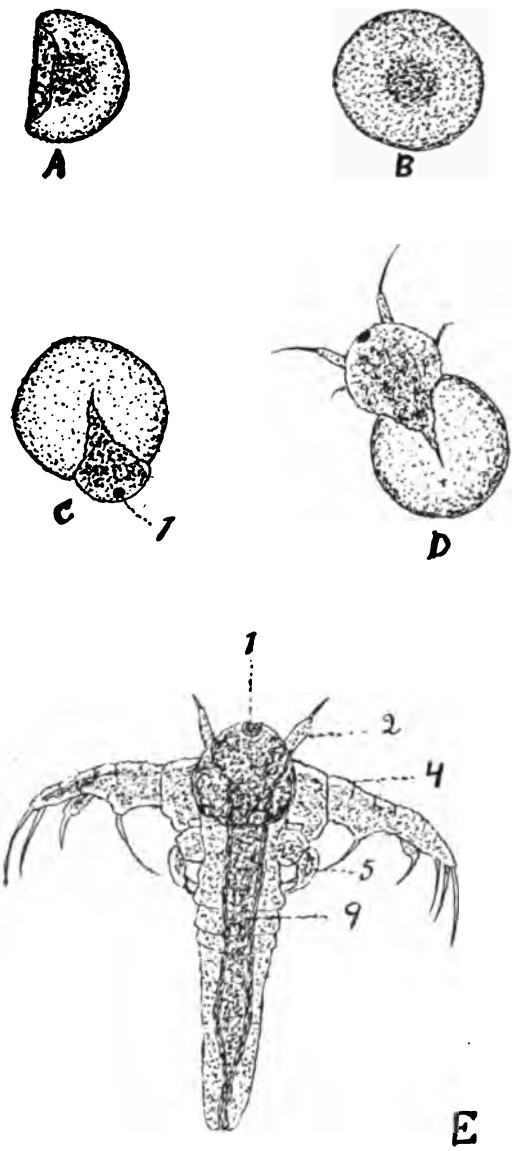

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PLATE IV.

Later nauplius stages.
Figures of plates III. and IV. drawn on the same scale.
Notations.

1. Ocellus.
2. First antenna.
3. Compound eye.
4. Second antenna.
5. Mandible.
6. Thoracic appendages.
7. Gills.
8. Ovisac.
9. Intestine.
10. Basal segment of the first antenna.
11. Distal segment of the first antenna.

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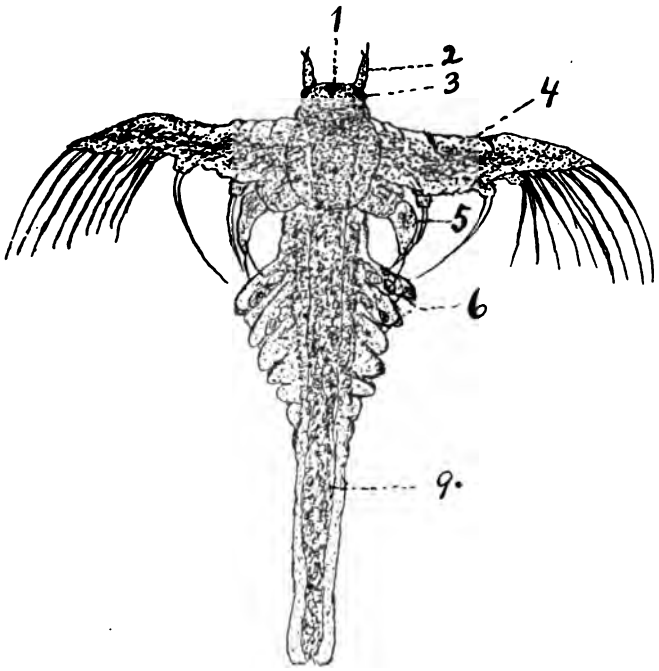

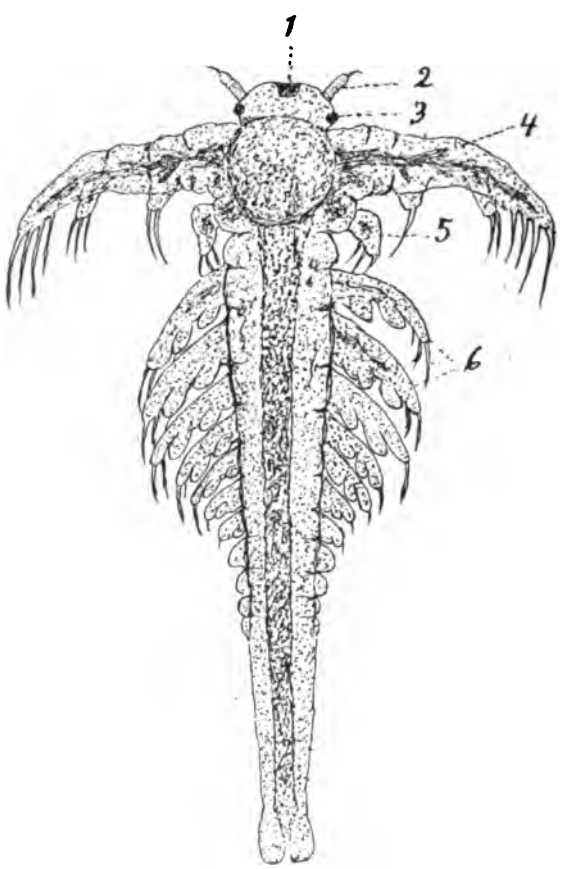

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THE RELATIONS BETWEEN THE INTERSTITIAL GLAND OF THE TESTICLE, SEMINIFEROUS TUBULES AND THE SECONDARY SEXUAL CHARACTERS.[1]

LEO LOEB.

The following observations may prove of interest in the analysis of the relations between male gonads and secondary sexual characters.

While, as is often the case, an experiment of nature, unaided by experiments in the laboratory, is unable to give a definite answer to the questions which it raises, yet it would at present be impossible to duplicate at will in the laboratory an experiment such as we observed and it is, therefore, desirable that it should be recorded.

Our observations were as follows:

Guinea pig No. 1523 had been sent to our laboratory by an animal breeder as a female that had apparently been in heat, but in some respects behaved abnormally. The report of the breeder was as follows:

"The female through her actions gave every sign of being in heat; but upon examining her, she did not look to me as if she really was in heat. I had her with two other females and she would mount them just the same as a male guinea pig would do when in the act of copulation and she would also allow the other females to act in the same way. She would stand in this position, meanwhile expanding the vagina, which generally is a sure sign of their being in heat. I would say that she is with young; at least she should be pregnant, because she had been with a male for some time. Expect some young at any time."

The breeder made his observations July 8, 1917. On July 12, 1917, the animal, which weighed 615 grams, was prepared for operation. Under ether anesthesia lumbar incisions were made,

[1] From the Department of Comparative Pathology, Wshhington University Medical School, St. Louis, Mo.

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in order to expose the ovaries. Typical ovaries were, however, not found. Instead, we encountered near the place where the ovaries usually are situated, but perhaps a little farther down, somewhat large and round, rather soft bodies, each about the size of a pea, surrounded by much fat tissue. On cutting through the center the color was found to be slightly yellowish-brown. From here some fibrous bands extended downward in the direction in which the uterine horns are usually situated. An organ that resembled the uterus was not found. The vagina was also lacking. Neither was a penis, vas deferens or a descended testicle visible.

The brownish red bodies as well as pieces of the fibrous bands were fixed in Zenker. Both of the bodies were completely cut into serial sections. Tissue from the places where the mammary glands are usually situated was also fixed and sectioned.

Microscopic examination:

1. The round bodies situated below the kidney were seen, microscopically, to consist of testicle tissue in which there was an extraordinarily marked development of interstitial gland. The testicle tubules were lined by one layer of epithelial cells, the outline of which was not very definite. Towards the center of the tubules they formed a network of fine fibrils; a sharply defined lumen therefore did not exist in the tubules. The cells had either a cuboidal or cylindrical form as far as their indefinite outline permitted of such a characterization. In some cases the whole protoplasm of the cell was drawn towards the center of the tubule in a tail-like structure. The nucleus was situated in the center of the cell and was very characteristic. It represented a clear vesicle in which one deep-staining large nucleolus was visible. Occasionally the nucleolus divided into two parts. In various places the tubules were lined with several cell rows. Sometimes the cytoplasm and the nucleus of one cell swelled, and such a cell encroached upon the territory of the neighboring cell, lying more towards the center of the tubule, pressing against it; and thus one cell surrounded, crescent like, the neighboring cell. Occasionally some cells degenerated, the nucleolus persisting longest, while the rest of the nucleus had already disappeared. Mitoses were seen quite frequently in these tubule cells and monasters as well as diasters

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were found. The tubules were enveloped by a circular layer of flat connective tissue cells, which were drawn out into long fibers with flat nuclei. Each tubule was surrounded by several, usually two to five, layers of interstitial cells. Each tubule with the surrounding concentrically arranged interstitial cells formed a group which was often quite sharply separated from neighboring similar groups through lymph vessels or lymph-spaces and blood vessels and accompanying connective tissue strands. In certain places larger blood vessels accompanied by lymph vessels were found in the tissue. Blood capillaries were numerous in certain places. Occasionally a tubule was found adjoining the connective tissue directly, without being separated from the latter by interstitial cells. The character of the interstitial cells varied somewhat in different parts of the organ. Usually they were large polygonal, partly rounded off or oval cells with a large vesicular nucleus, in which several small nucleoli were generally seen, instead of the one clear nucleus which was so characteristic of the tubule cells. The nucleus in the interstitial cell was not so light and transparent as in the tubule cell. The sizes of the interstitial cells varied. The cytoplasm became finely vacuolar, at first in the periphery. In the center there was still a solid material staining red with eosin. Later the whole cytoplasm became finely vacuolar. This vacuolization was usually accompanied by a considerable increase in size of the cells. In some cases the vacuoles enlarged still more, the walls separating neighboring vacuoles disappeared and irregular cavities became visible in the cells. In those cases the nucleus became irregular and shrank, and when this last stage had been reached, the cell was evidently degenerating. Many finely vacuolar enlarged cells appeared otherwise normal and viable. These finely vacuolar, swollen cells occurred especially around the lymph vessels, or perhaps also around certain blood vessels. We must assume that substances reaching the cells from the circulation were deposited within the cells and caused the vacuolization, the nearness to the source of this substance determining the frequency and intensity with which these changes took place. Under the same conditions the accompanying fibrous tissue became edematous. Apparently the vacuolization of the interstitial cells and the edema-

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tous state of the stroma are analogous phenomena. We were only rarely able to find mitoses in the interstitial cells. In this respect the latter differed from the tubule cells in which mitoses were quite frequent. There could be found at one end of each testicle a system of branching epithelial ducts, with a narrow lumen. They were lined by a layer of small and densely packed, usually cuboidal or flat epithelial cells, with nuclei which filled the greater part of the cells. In these cells either one or several nucleoli were found. If one nucleolus was present the nucleus resembled that of the tubule cells, except that it was smaller. At other places the lining cells and nucleus were low cylindrical. In the lumen of these ducts not infrequently some invaginations of the epithelium were found. On the one side the ducts formed a union with the tubule of the testicle. Between the two we found transitional structures consisting in part of tubular and in part of duct epithelium. At the other end the ducts entered the fat tissue which surrounded the testicle and made connection with some larger ducts. One change that took place in a number of tubules needs special mention: A few or many, at some places even the majority of the tubule cells underwent certain changes which made them very similar to interstitial cells. They enlarged, became polygonal or somewhat round, and their cytoplasm stained more strongly with eosin. The cytoplasm became finely vacuolar, at first the periphery and later the greater part of the cell undergoing this change. At the same time the nucleoli divided into several particles which were dispersed in the nucleus. The nucleus became a round vesicle which had lost the transparency so characteristic of the nucleus of the tubule cells. Accompanying this change the layer of flat connective tissue cells which separated tubules and interstitial cells disappeared and at various places interstitial cells adjoined directly the tubule cells, both kinds having become very similar. Such changes seemed to be frequent especially in places where the interstitial cells were swollen and had become finely vacuolar. Three interpretations of this condition suggest themselves:

1. As a result of their enlargement the interstitial cells push into the tubules, and thus the appearance within the tubules of typical tubule cells adjoining directly cells resembling interstitial

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cells might be explained. At the same time the enlarged interstitial cells exerted a pressure on the tubule cells which made them crowd closer together than usual. This interpretation is not admissible, because the cells within the tubule began to swell at a time when the connective tissue layer separating tubule and interstitial tissue was not yet broken through. At first the enlarging cells still possessed the nucleus characteristic of the tubule cells and only gradually the character of the nucleus changed concomitantly with the later stages of the transformation.

2. It might be assumed that an actual transformation of tubule tissue into interstitial tissue took place, and that the gland-like interstitial tissue was merely further differentiated tubular gland tissue. Many pictures seemed to suggest such an interpretation. However, the fact that in many other places, especially where the interstitial cells were still solid and smaller, and, therefore, younger, the line of demarkation between tubule and interstitial tissue throughout was very sharp made, after all, this interpretation improbable. Furthermore, it would be contradicted by what is known of the origin of the interstitial cells.

3. It is most probable that the same factors which produce the changes in the interstitial cells, at the same time called forth similar changes in the tubule cells. Probably certain substances furnished by the lymph or blood vessels were simultaneously responsible for both sets of changes. In favor of this interpretation might be cited the fact that we did not usually find in the tubules that extreme vacuolization, which was present in some of the interstitial cells. We are, therefore, inclined to adopt the third interpretation.

The round bodies which we found near the place where normally the ovaries are situated therefore represent undescended testicles. There is a system of ducts leading from the testicle tubules to the surrounding fat tissue. The tubules are relatively simple structures in which spermatogenesis does not take place. The epithelium lining them corresponds to the sustentacular cells of the normal testis. There is present an extremely marked development of the interstitial cells. The structure of this testicle differs, therefore, markedly from the normal testicle of the

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guinea pig which consists principally of tubules with several layers of epithelium which are in the process of spermatogenesis. Between these tubules there is very little stroma with blood vessels and a very small number of polygonal cells.

Sections through the tissue, taken from the place where normally the uterus is situated, show fibrillar connective tissue in which are situated nests composed of rather large polygonal, cylindrical or ellipitical cells, the protoplasm of which stains well with eosin. The nuclei of these cells are round vesicle with chromatic particles distributed rather diffusely. These cells form the matrix of the neighboring fibrillar connective tissue. Through rarefaction of the cytoplasm and through a condensation, backing together of the nuclear chromatin these cells are transformed into the surrounding connective tissue. We also studied in microscopic sections the fat tissue which filled the space between the undescended testicle and the kidney, without finding any trace of further testicular or of ovarian tissue.

Mammary Gland.—The nipples of the guinea pig were distinct and we cut out pieces from the tissue where normally the mammary gland is situated. In the male a few scattered ducts lined by epithelium in which mitoses do not usually occur represent the mammary gland.[1] In our case, a very much furthergoing development of the mammary gland was found on microscopical examination. Sections through one of the two mammary glands showed solid tissue consisting of a large number of lobules of mammary gland tissue, joined together by thin strands of fibrous tissue. The whole gland was surrounded by fat. Each lobule consisted of acini lined by cuboidal epithelium of medium size. The nuclei were vesicular and relatively large. The lumen of the acini varied in size and often contained some colloid material or desquamated cells. Occasionally an acinus cell was found to be vacuolar. In the stroma between the acini there were numerous small connective tissue cells. At some places, where the interstices between the acini were somewhat wider and the stroma was slightly edematous, polynuclear leucocytes staining deeply with eosin were found. The largest ducts were

[1] In the ducts of the mammary gland of a young male guinea pig, about 30 days old, we encountered however one mitosis.

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as usual surrounded by denser fibrous tissue. At one pole of the gland a system of ducts and acini lined by higher and larger epithelium extended into the surrounding fat tissue. The lumen of these ducts and acini was wider. Mitoses could not be seen in the mammary gland. The mammary gland of the other side, which was also surrounded by fat, was smaller, at least the amount of gland tissue found on sections, which had been through different parts of the piece, was less; but it still surpassed considerably that found in normal animals. Furthermore, the acini were composed of high cylindrical cells, surrounding a rather large lumen. There were a few desquamated cells in the gland lumina, the acini being arranged around the larger ducts. Here and there mitoses were seen in the acini. The stroma between the acini was loose in texture and contained a number of fibroblasts.

Discussion.—The following are the chief points of interest in these observations:

1. We have in this case apparently to deal with an undescended testicle in an adult guinea pig. Such testicles have been observed in man and several species of animal, especially in the horse and pig. This case is an example of the same condition in the rodentia. Common to all cases which have been so far described is an imperfect development of the seminiferous tubules and a hypertrophy or a hyperplasia of the interstitial cells of the testicle. As to the literature concerning such cases we may refer especially to Bouin and Ancel (1) Tandler and Grosz (2), and Whitehead (3). Both of the features we mentioned are present in a marked degree in our case. Spermatogonia are completely absent; the tubules consist entirely of Sertoli cells. The retention of the testicle acted in a way comparable to the ligation of the vas deferens which brings about similar results which are, however, usually not quite so far going. In addition, it is very probable that the condition which prevented the normal descent of the testicle was directly responsible for the lack of development of spermatogonia. As to the overdevelopment of the interstitial gland, in those cases, we may conclude that it stands in some causal relation and is subsequent to the underdevelopment of the seminiferous tubules. In various other conditions in which atrophy

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or underdevelopment of the tubules has been observed, or produced experimentally (ligation of vas deferens, Roentgen ray injury of the testicle (4) (5) and certain diseases) a hypertrophy or a hyperplastic condition of the interstitial gland has been noted. Herxheimer and Hoffman (5) suggest that this hyperplasia is comparable to the substitutive growth of connective tissue which takes place whenever parenchymatous elements are destroyed. This explanation, however, does not seem to take into account the principal variations which have been observed in the condition of the interstitial gland. Bouin and Ancel (1) have shown that in the horse the interstitial gland is present in the fetus; it degenerates in the immature animal and begins anew to develop at the time of puberty. In the sexually mature animal there seems to exist a certain antagonism between the activity of the seminiferous tubules and the interstitial gland. The interstitial gland becomes hyperplastic in cases in which the tubules decrease in activity and vice versa. Under the conditions of tubular atrophy or degeneration which we named above the interstitial gland hypertrophies, and according to Tandler and Grosz in the mole, in which sexual activity follows a yearly cycle, the interstitial gland shows the greatest development at a time when the seminiferous tubules are least active. In hibernating animals the interstitial gland decreases at the period of general metabolic inactivity (von Hansemann); similarly in cases of very pronounced general undernourishment, or of very marked degenerative processes in the testicle the interstitial gland suffers and may disappear. Some observations indicate, furthermore, that under certain conditions extirpation of one testicle may lead to a hypertrophy of the interstitial gland in the second one. All these facts can evidently not be explained otherwise than by assuming that there are several factors active and that chemical and not merely mechanical substitutive factors play a rôle in the regulation of the growth of the interstitial gland. It seems to be a general function of gland and other epithelial cells to stimulate through their activity the surrounding stroma. We have described such an occurrence especially in connection with the cyclic growth of the mammary gland, but it seems to be a phenomenon of general significance. In a similar way we may as-

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sume that growth processes in the seminiferous tubules stimulate the activity of the connective tissue cells and those specialized connective tissue cells which have the potentiality of becoming transformed into interstitial gland. This would explain the development of the interstitial gland during embryonic development and at the time of puberty. We might designate this as the concomitant growth of the interstitial gland. In order to explain the second kind of growth in the interstitial gland, which we might call the "alternative growth," we must assume that in a certain way the activity of the sexually active seminiferous tubules exerts an inhibiting effect on the growth of the interstitial gland and that a diminution of this inhibiting effect leads to the "alternative" growth of the interstitial gland. An analogous condition is found in the rabbit ovary in which the atresia of certain follicles leads to a secondary gland-like development of the theca interna. How far such an equilibrized condition is maintained through chemical, how far through finely adjusted mechanical factors, we do not know at present. However, in the somewhat analogous case of the transformation of the connective tissue cells of the uterine mucosa into decidua, we have analyzed the underlying factors experimentally and found that a combination of specific chemical and mechanical factors, which latter are likewise to a certain extent specific, is responsible for this change (6).

In addition, the interstitial cells of the testicle are accessible to that chemical stimulation which is implied in compensatory hypertrophy and they are furthermore affected by lack of common foodstuffs.

2. The specific effects exerted by the interstitial cells have been ascribed by Bouin and Ancel to certain products formed within the cells which microchemically behave like fats or lipoids. We found, in our case, reasons for assuming that drop-like substances were furnished the interstitial cells from the surrounding lymphatics and perhaps also from the blood capillaries. How far the substances taken up from the circulation and perhaps modified within the cells are identical with the substances described by Bouin and Ancel and Whitehead, we cannot at present decide.

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3. Various writers, especially Bouin and Ancel, concluded that the interstitial cells of the testicle are responsible for the development of sexual desire in the male. Our observations seem to confirm this view. The complete absence of spermatogonia in the tubules, together with the marked development of the interstitial gland was accompanied by the presence of marked manifestations of sexual instinct.

4. Bouin and Ancel, Steinach (7, 8) and Tandler and Grosz not only ascribe to the interstitial gland the function to cause chemically those nervous changes which find expression as sexual desire, but the additional task of enhancing the development of the male sexual characters, of allowing the full development of prostate, penis, seminal vesicle, and of suppressing the development of the female secondary characters. Our observations apparently contradict these conclusions. In our case, the very marked development of the interstitial gland was accompanied by the absence of penis, seminal vesicles (and presumably of prostate), and on the other hand was associated with a very marked development of the mammary gland. The latter resembled a proliferating breast, a "proliferating gland" such as Dr. Hesselberg and the writer found it in certain stages of the sexual cycle in the female guinea pig (9). Our observations prove that the presence of a very strongly developed interstitial gland is perfectly compatible with the existence of certain female, and with the absence of certain male secondary sexual characters. A priori two interpretations of this fact are possible:

(*a*) The interstitial gland of the testicle has not a specific function in the sense assumed especially by Bouin and Ancel and Steinach. (*b*) The interstitial gland is specifically "male enhancing" in its function, but the end effect does not only depend upon this specific action of the interstitial gland, but also, and primarily so, on the system on which it acts. We have reason for assuming that in a certain respect an individual can be compared to a more or less sensitive balance in his or her sexual potentialities and that different individuals of a certain species differ in the resting point of their sexual equilibrium (10, 11, 12, 13, 14). Without the interference of hormones which are produced by the gonads, the point of equilibrium in some individuals

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would be neutral; in others it would be deviating more or less in the direction toward the male; in still others toward the female side. On the whole the male would usually deviate to the male and females to the female side. However, differences exist in this respect in different classes and species of animals and also in individuals of the same species. There are, furthermore, differences in the relative degree of stability of this point of equilibrium. In insects it is apparently very stable and little or not at all influenced by hormones of gonads; in mammals the equilibrium is labile and readily influenced by hormones of the gonads. In mammals, the male hormones can be compared to weights added to the male side of the scales and female hormones influence the scales in the opposite direction. It seems probable that in our case we had to deal with an individual with imperfect male gonads which represented a system with a very slight tendency towards the male side and with a relatively strong tendency toward the female side. In such an individual with tendency toward female secondary sexual characters even a strong male hormone is not able to prevent the growth of the mammary gland, and this female tendency is probably in some way connected with the suppression of male secondary characters during embryonic development, and perhaps also directly or indirectly with the failure of the testicle to descend to its proper place and develop perfectly. In such a relatively stable system tending toward femaleness in its secondary characters, even a specific male hormone, such as is supplied by the interstitial gland of the testicle, has little chance to become effective. Whether, under certain conditions, the hormone given off by the interstitial gland of the testicle might in addition be able to promote growth of the mammary gland, cannot be decided on the strength of the evidence which we have at present.

Summary.

1. It is shown that in a guinea pig with undescended testicle, in which spermatogonia were lacking, and the interstitial gland was hypertrophic, sexual desire was strongly developed.

2. An attempt is made to analyze the various factors that determine the growth of the interstitial gland in the testicle.

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3. It is shown that, notwithstanding the presence of a hypertrophic interstitial gland, there was an absence of male secondary sexual characters and that the mammary gland had a female character. A provisional interpretation of these facts is given on the basis of our present knowledge of sex determination.

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Additional Note

The foregoing paper was written without the knowledge of two quite recent, very important publications by F. R. Lillie and C. L. Chapin, which may have some bearing on our observations, and which it will therefore be necessary to briefly discuss from

[1] F. R. Lillie, *Journal Exper. Zoölogy*, 1917, XXIII., 371. C. L. Chapin, *ibid.* 453. I am much indebted to Prof. Lillie for calling my attention to these papers,

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this point of view. In the first of these two papers Lillie gives a detailed account of the anatomy of the internal and external genital organs in freemartins in various stages of embryonic development and of his interpretation of the cause of the abnormalities and in the second paper Chapin describes the microscopical findings in these cases. It follows from Lillie's observations and analysis that the sterile freemartin represents an individual which was originally a female, but in which under the influence of hormones carried into her circulation from the chorionic vessels of a male twin, the female characteristics were more or less suppressed and male characters acquired. This not only applies to the secondary sexual characters, but even to the structure of the gonads. As Chapin has shown, the development of the ovarian cortex and of ovarian follicles is more or less interfered with or may be altogether suppressed, and the medullary sex cords develop often in a way similar to the seminiferous tubules, although spermatocytes or spermatozoa to not seem to be produced. The development of the Müllerian ducts is likewise interfered with and seminal vesicles may develop instead. The external genitalia and the mammary gland are least affected by the male forming substances, which according to Lillie originated in the testicle of the male twin and were from here transmitted to the female twin; these latter organs remain usually female in character.

These important findings may have some bearing on the interpretation of our observation. There exist undoubtedly marked similarities between the condition found in the freemartins and in the condition of the genital organs of the guinea pig which we examined. However, while Lillie and Chapin examined embryological material, we have to deal with an adult animal.[1] In our case we observed a gonad which shows the characteristics found in testicles under various pathological conditions. Similar changes have been produced in testicles experimentally under the influence of Roentgen rays. The tubules, the character of the cells composing them and particularly of the nuclei closely resemble structures which we found after transplantation of

[1] Microscopic sections through the gonads of an older freemartin from the material of Dr. Lillie show a great similarity with our sections through the gonads of the guinea pig.

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testicle and epididymis in the guinea pig. The interstitial cells are developed to an extraordinary extent. The organs were cut in complete serial sections and no trace of ovarian structure was found. From the descriptions given by Miss Chapin, I should judge that a similar condition has not been observed in the fetal gonads of the freemartins. In the guinea pig, uterus and vagina as well as vas deferens, seminal vesicles and penis were absent. While I was unable to find a description of the histology of the mammary gland in freemartins, we observed in our case a typical female mammary gland with the added peculiarity that the mammary glands of both sides were unequal.

The question arises, whether the guinea pig which we observed was an analogue to the freemartin in cattle. Was this animal originally the twin sister of a brother, under whose influence the observed deficiencies were produced? While we cannot definitely exclude such a possibility we must consider the fact that neither ourselves nor anyone else has so far observed twins with a common chorion and deficiencies in the development of the sexual organs in rodents. Lillie himself points out the great improbability of such an occurrence in animals in which the ova enter the uterine wall at an early date and in which the uterine horns are separated. On the other hand, we accidentally observed in several cases absence of one uterine horn in guinea pigs. Malformations of the sexual organs do therefore occur. It is very probable that similar malformations may be due to different interferences in the embryo, just as we know that cyclopic conditions of the eye can be produced in different ways. We interpreted in our paper the conditions which we found in the guinea pig as consisting essentially in the presence of an abnormal, incompletely developed male gonad in which generative cells are absent, and in which the interstitial cells show an unusually pronounced development. This maleness of the gonad and especially the marked development of an interstitial gland is associated with deficiencies in the formation of the efferent genital ducts and with a female mammary gland. We interpreted this unusual condition as due to the original female constitution in the animal, to the tendencies toward femaleness which prevented the male interstitial cells from exerting their specific influences

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on the mammary gland, If this guinea pig should represent an analogue to the freemartin in cattle, we would have to assume that the male characteristics of the gonad were produced in an original female through the influence of the testicular substance of the male brother. While such an origin has been made possible through the findings of Lillie, the essential point is that, whatever the origin of this condition, we have every reason to consider the gonad which we described as male.

It is the complete examination of the gonads in serial sections which makes it possible for us to exclude the presence of ovarian structures in this animal and to prove the similarity of the organs with the testicles found under certain experimental and natural pathological conditions. There is no reason for assuming that the gonads in our case represented really ovarian tissue. We have examined many hundred of ovaries of guinea pigs of various ages and have never been able to detect in the ovaries of guinea pigs structures resembling the interstitial gland of the testicle, while on the other hand such structures are strongly developed in testicles which have been retained in the abdominal cavity.

The findings of Lillie and Chapin suggest very strongly, and I take this also to be the interpretation of Lillie, that even in the freemartin the gonad which originally was destined to become female, assumes at least in part true male characters. If this were not so, it would be difficult to explain why seminal vesicles could develop in a freemartin. We should expect that the autogenous ovarian substances would prevent the heterogenous testicular substances from exerting such an influence on the secondary sexual organs of the female twin.

We found in our case a marked development of sexual instinct associated with the absence of generative cells and with a very marked development of the interstitial tissue. Now, as we have pointed out in previous papers, it is improbable that in the guinea pig the interstitial gland is responsible for the manifestation of sexual desire in the female.[1]

[1] Loeb, Leo, *Zentralblatt f. Physiologie*, 1911, XXV., No. 9, and Loeb, Leo, "The Relation of the Ovary to the Uterus and Mammary Gland from the Experimental Aspect." *Surgery, Gynecology and Obstetrics*, 1917, XXV., p. 300. (A review of the literature concerning this and other aspects of the mechanism of the sexual cycle.) In accordance with the most widely accepted view we assumed in our

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Typical interstitial cells are absent in the normal ovary of the guinea pig. If we should assume the gonads in this guinea pig to be female our own observation would constitute a decisive proof that also in the female guinea pig the interstitial cells produce the substance which is responsible for the sexual desire—a conclusion which, however, as we stated above, would be contradictory to other facts which we established previously.

The case which we described may be compared to a single equation communicated by nature, in which two variables are present. While one equation is not sufficient to determine the unknown factors in such a case, the variables have at least been carefully defined in our case. We may hope that later a second equation will be found which will permit defining all the variables. We thought it therefore of interest that these observations should be published.

discussion that it is the so-called interstitial gland of the testicle which is responsible for the development of the secondary sexual characters and of sexual desire in the male. We wish, however, to emphasize the fact that in our case epithelial elements were present in the testicle in addition to the interstitial gland and apriori we cannot exclude the possibility that they and not the interstitial gland are the causative agency in determining sexual phenomena. We are likewise not certain that this can be excluded in those experimental and pathological conditions in which the changes were attributed to the interstitial gland. However we are not directly concerned with this question and we are not in a position to contribute definite data which would favor or contradict this view. We wish, however, to reiterate the fact that on various occasions we made observations which do not seem to accord with the assumption that in the ovary the so-called interstitial gland initiates heat.

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PHYSIOLOGICAL SENESCENCE IN HYDROMEDUSÆ.

C. M. CHILD.

The following observations on senescence in the hydromedusæ were made at the Puget Sound Marine Station, Friday Harbor, Wash., during the summer of 1917. I wish to acknowledge my obligation to the director of the laboratory, Dr. Frye, for the use of a room and the privileges of the station, and for his kindness in providing special glassware and other equipment. To other members of the staff I am also deeply indebted for many courtesies.

The great abundance, variety and large size of hydromedusæ at Friday Harbor makes this an extremely favorable locality for work on these forms. During the summer of 1917 five species of hydromedusæ, *Phialidium gregarium*, *Æquorea cærulescens*, *Sarsia rosaria*, *Mitrocoma discoidea* and *Stomatoca atra* could readily be obtained in considerable numbers, often by the hundred, from the laboratory float, and five other species of other genera were more or less abundant, besides two species of siphonophores. The first four species named above furnished the material for the observations and experiments on ageing, and the results are essentially the same in all.

The work was done entirely on free-swimming medusæ and is essentially a comparison of younger and older individuals, no attempt having been made to obtain the medusa-bud stages before detachment from the hydroid.[1] In the selection of individuals of different age for experiment, size of the animal and degree of development of the gonads were used as superficial criteria of age. As might be expected, these two criteria are in general in close agreement, for since the range in variation in size of the medusæ-bud at a given stage of development is not very great, a larger medusa must in general have undergone a greater amount of growth than a smaller, and may therefore be

[1] In *Pennaria* a progressive change in physiological condition has been observed from earlier to later stages of medusa-buds (Child, '15, pp. 150–152).

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expected, if age differences exist, to be physiologically older. As has been shown for *Planaria*, and other simple animals (Child, '15, Chapter IV.—VII.) physiological age is not necessarily a function of the length of time which the individual has lived, for nutrition, agamic reproduction and various other factors play a rôle in determining the physiological age at any given time. Size is therefore a far more adequate criterion of age in such forms than time. Moreover, since the development of the gonads in medusæ progresses from a relatively early stage to sexual maturity the condition of the gonads also serves as a measure of the stage in its life history which the animal has reached.

The chief method employed was that of determining the susceptibility of younger and older animals to various agents. The general relation between susceptibility and age has been discussed elsewhere (Child, '14, '15) and need only be briefly restated. In general, in concentrations or intensities sufficiently high to kill without permitting acclimation, the susceptibility decreases with advancing age. In low concentrations, which permit some degree of acclimation, the susceptibility increases with advancing age because the young individual possesses a greater capacity for acclimation then the older, therefore becomes more rapidly and more completely acclimated and so dies later than the older, or lives indefinitely, while the older dies sooner or later.

It has been shown that a relation exists between susceptibility and metabolic activity, more particularly the oxidative or energy-freeing reactions. In general, susceptibility to the higher concentrations or intensities varies directly, and susceptibility to the lower concentrations varies inversely as the rate of these reactions, because acclimation occurs more rapidly and more completely where metabolic activity is greater. This relation between susceptibility and metabolic condition has been shown to hold good not only for different individuals, *e. g.*, young and old (Child, '15) but for different regions of the body of a single individual (Child, '16*a*).

It does not follow, however, from the existence of a relation between susceptibility and rate of metabolism or of certain metabolic reactions, that all agents for which such a relation

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exists act directly upon the chemical reactions or that they all act in the same way. Much remains to be learned concerning the nature of the action of external agents on living protoplasm, but the fact is already clear that protoplasmic structure, aggregate condition, permeability, surface tension, etc., are associated with metabolic condition. Susceptibility to a given agent serves at best merely as an indicator of protoplasmic and metabolic condition and tells us nothing concerning the way in which the agent acts. As our knowledge of susceptibility to different agents and conditions increases, we find that under certain conditions different agents give us different results, and such differences in susceptibility relations often serves to throw additional light on the physiological condition of the protoplasm or the action of the agent, or both. The general relations stated above hold for a wide range of agents and conditions, and it is primarily with these general relations that we are concerned in the present paper.

Age-differences in Behavior.

A very conspicuous difference between young and old individuals is the much higher rate of pulsation in the young animal and its progressive decrease with advancing age. Table I. gives characteristic data on this point.

Table I.

Species.	Diameter of Body in Mm.	Pulsations in 20 Ser. at 14° C.
Æquorea cærulescens	7	40
	20	32
	50	20
Mitrocoma discoidea	18	28
	60	16
Sarsia rosaria	5	40
	10	30

In every species observed, ten in all, a similar difference in the rate of pulsation exists, the rate in the sexually mature animal being in most cases about half that of the animal at the beginning or in very early stages of gonad development. By repeated stimulation the rate may often be somewhat increased, particularly in old animals, but the differences in animals under ordinary conditions are of the order of magnitude indicated. This dif-

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ference in pulsation rate in itself indicates a very marked decrease in metabolic rate with advancing age. Mayer ('06) has noted that in the scyphomedusa *Cassiopeae* the rate of pulsation decreased with advancing age.

Young and old animals also differ in their behavior in another way. Under the usual conditions periods consisting of a larger or smaller number of pulsations alternate with periods of rest. The total length of pulsation periods as compared with rest periods decreases with advancing age; in other words the younger animal spends a larger portion of the time than the older in rhythmic pulsation. Pulsation periods may either be longer or more frequent or both in the younger animal than in the older. In *Æquorea* this difference is perhaps even more strongly marked than in other forms observed. In the large, sexually mature animals 60–80 mm. in diameter, when under natural and so far as possible constant conditions, the periods of quiescence are often one or two minutes long, sometimes even longer, the animal drifting passively during this time without a single pulsation. Following such a quiescent period a single pulsation or a pulsation period may occur. Under the same conditions the quiescent periods in young animals 15–20 mm. in diameter are usually very much shorter, commonly only a few seconds and the pulsation periods are usually longer than in the old animal. The young animal is then more continuously active and gives the impression of a much greater degree of "spontaneity," since it is usually impossible to distinguish any external exciting factor responsible for the beginning of pulsation after a period of quiescence. In the other species observed similar differences between young and old exist, but periods of quiescence are usually shorter than in *Æquorea*.

The irritability of the young animal as indicated by the effectiveness of direct mechanical stimulation in inducing pulsation during a quiescent period is distinctly greater than that of the old. Usually the quiescent animal in the earliest stages of gonad-development responds by pulsation to the slightest touch in the marginal region, while the sexually mature animals commonly respond only to much more intense mechanical action.

These differences between younger and older medusæ show of

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course a considerable range of variation in different individuals, but are nevertheless striking features of the behavior of these animals when attention is directed to them and they remind one irresistibly of age differences in behavior in much higher animals. The apparent restlessness and spontaneity, the more rapid pulsation rate, and the greater irritability of the young animal as compared with the old all suggest that in these, as in other organisms, so for as known, the life history of the individual is from an early stage a process of physiological senescence which expresses itself dynamically as a decrease in rate or intensity of the fundamental energy-liberating metabolic reactions.

Susceptibility.

In determining the susceptibility of the animals various other criteria besides death and disintegration may be used as a check, viz., cessation of rhythmic pulsation, the disappearance of muscular contractility and shrinkage of jelly in some agents. As regards the relation between susceptibility and age all of these criteria give the same results. It is usually difficult or impossible to determine the exact time when an animal ceases to respond to stimulation by rhythmic pulsation or by slow muscular contractions and the early stages of shrinkage and of disintegration are not less difficult to determine. The times given in the following tables represent approximations only. Frequently pulsation or contractility is present at one observation and absent at the next, and the time of its disappearance can only be estimated. Nevertheless there is no difficulty in distinguishing the differences in susceptibility between young and old animals. The experiments were mostly performed with single pairs, one young, one old, but in some cases two or three of each were used.

Æquorea cærulescens.

This, being one of the most abundant species, was used to a large extent. The smallest and youngest individuals found were 8–10 mm. in diameter, without gonads, the largest, and oldest, sexually mature animals 60–75 mm. in diameter. The chief results are tabulated below. In these tables the sign ± following a given time indicates that this is estimated from two observa-

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tions between which the change in question occurred, and . . . ? indicates that the change or process did not begin until after the close of the experiment. In this species cessation of pulsation, disintegration and in some cases loss of contractility are available as criteria of susceptibility. The jelly is very firm and does not shrink to any great degree in any agent used.

The three tables agree in showing a greater susceptibility in young than in old animals, as indicated by cessation of rhythmic pulsation, loss of muscular contractility and disintegration. Special attention must be called to certain points. In Table II.

TABLE II.

Æquorea: SUSCEPTIBILITY TO KNC.

Series.		Diameter in Mm.	Conc. KNC.	Duration of Experiment.	No Pulsation after Stimulation.	No Contraction after Stimulation.	Disintegration.
1	*A* . .	7	*m*/10000 After neutral red	1 hr.	1 min. ±	5 min. ±	. . .?
	B . .	12			5 "	10 " ±	. . .?
	C . .	20			10 "	20 "	. . .?
	D . .	50			40 "	60 " ±	. . .?
2	*A* . .	12	*m*/10000	4 hrs.	30 sec.		. . .?
	B . .	30			20 min.		. . .?
7	I. *A* . .	18	*m*/500	17 hrs.	2–5 min.		12 hrs. ±
	I. *B* . .	60			10–15 "		17 " ±
	II. *A* . .	18	*m*/200	6 hrs.	3 min.		4–6 hrs.
	II. *B* . .	60			8 "		6–? "
	III. *A* . .	16	*m*/100	2 hrs.	At once		1 hr.
	III. *B* . .	70			" "		2 "
	IV. *A* . .	20	*m*/100	3 hrs.	At once		$1\frac{1}{4}$–$1\frac{3}{4}$ hrs.
	IV. *B* . .	60			" "		$1\frac{1}{2}$–3 "

it is of interest to note that KNC *m*/10000 is about as effective, or in some cases more effective than much higher concentrations, *m*/500, *m*/200, in stopping pulsation. It has been observed in other cases (Child, '16*a*, '16*b*) that rapidity of action of KNC and certain other agents, as indicated by death or other effects, increases only slightly with increase in concentration above a

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certain limit or may even decrease. The facts suggest that the high concentrations may decrease permeability to themselves, at least up to a certain limit where their action becomes violently destructive to protoplasm, and so practically instantaneous.

In Table III., HCl and Table IV. KOH, the action in stopping pulsation increases very rapidly with increasing concentration,

TABLE III.

Æquorea: SUSCEPTIBILITY TO HCl.

Series.		Diameter in Mm.	Conc. HCl.	Duration of Experiment.	No Pulsation after Stimulation.	No Contraction after Stimulation.	Disintegration.
II.	*A*..	18	*m*/1000	5 days	4½ days	...?	...?
	B..	60			...?	...?	...?
III.	*A*..	15	*m*/1000	5 days	3 days −	4 days +	...?
	B..	40			...?	...?	...?
3 IV.	*A*..	18	*m*/800	5 days	3 days +	4 days ±	...?
	B..	60			...?	...?	...?
V.	*A*..	18	*m*/500	21 hrs.	At once		8–12 hrs.
	B..	60			1 min. ±		17 hrs. ...?
4	*A*..	18	*m*/500	1 hr.	At once		
	B..	60			2 min.		
6	*A*..	18	*m*/400	1 hr.	At once		55 min. ..?
	B..	60			" "		...?
I.	*A*..	18	*m*/300	2¼ hrs.	At once		55 min. ..?
	B..	60			" "		2¼ hrs. ..?
5 II.	*A*..	18	*m*/300	2¼ hrs.	At once		
	B..	60			1 min.		

proportionately much more rapidly than the concentration, because more or less acclimation to the lower concentration occurs. In HCl *m*/1500—not tabulated—acclimation occurs in the young animal to a greater degree than in the old. After five days in this concentration the young animal still shows well-marked rhythmic pulsation after mechanical excitation while

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the old animal cannot be induced to pulsate. In concentrations of *m*/1000 or higher, the young animal is always the more susceptible (see Table III.).

The records on the loss of muscular contractility are very incomplete, but it is evident that rhythmic pulsation and general

TABLE IV.

Æquorea: SUSCEPTIBILITY TO KOH.

Series.		Diameter in Mm.	Conc. KOH.	Duration of Experiment.	No Pulsation after Stimulation.	Disintegration.
9 IV.	*A*..	16	*m*/1000	84 hrs.	12 hrs. ±	48 hrs. ±
	B..	60			48 hrs. ±	...?
8 I.	*A*..	18	*m*/800	34 hrs.	10 min.	4–6 hrs.
	B..	60			24 hrs.	...?
8 IV.	*A*..	15	*m*/800	28½ hrs.	5 min.	3–7 hrs.
	B..	60			26 hrs. ±	28½ hrs. ...?
9 III.	*A*..	14	*m*/600	9 hrs.	2 min.	4–6 hrs.
	B..	60			4–5 min.	9 hrs. ...?
9 II.	*A*..	18	*m*/400	9 hrs.	30 sec.	2–4 hrs.
	B..	75			1 min. +	3–5 hrs. +
9 I.	*A*..	18	*m*/300	5 hrs.	5 sec. ±	1–2 hrs.
	B..	70			20 sec. ±	3–5 hrs.
8 II.	*A*..	14	*m*/200	5 hrs.	At once	2–4 hrs.
	B..	60			10–20 sec.	3–5 hrs.
8 III.	*A*..	16	*m*/200	3½ hrs.	At once	1¼–2¼ hrs.
	B..	60			20–30 sec.	2–3½ hrs.

muscular contraction are not the same thing. Contractility of the muscles of the umbrella persists in all three agents long after ability to pulsate has disappeared, except in concentrations so high that death is practically instantaneous.

A complete but temporary inhibition of pulsation lasting from a few seconds to several hours occurs in almost all cases in HCl *m*/600. Above *m*/600 inhibition is usually permanent. At

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the lower limit of concentration for temporary inhibition only the young member of a pair is inhibited; with somewhat higher concentration both are inhibited for a short time but the young animal usually resumes pulsation before the old if the differences in age are not too extreme. As the concentration increases, the length of the inhibition period increases more rapidly in the young than in the old, until about the upper limit permanent inhibition occurs at once in the young animal while some slight pulsation may reappear sooner or later in the old. In short the age differences in susceptibility appear here as in other features of the action of external agents.

Experiments on recovery after temporary exposure to an agent showed that the relation between recovery and age is the same as in other forms examined. The reappearance of the pulsation response to stimulation is the criterion of recovery used. Where the concentration is not too high, the exposure too long or the difference in age between the animals too great, the young will recover before the old on return to water, but with higher concentrations longer exposures and greater differences in age the old animal recovers earlier and usually more completely than the young. These relations between recovery, age, concentration and period of exposure are of course merely a special case of the age-susceptibility relation. To higher concentrations or longer times of exposure the young animal is more susceptible than the old, but to low concentrations or short times of exposure it is able to adjust itself more rapidly or to recover more rapidly afterward than the old (Child '15, '16*a*). The degree of difference between young and old animals also differs widely with different agents, because acclimation to some agents occurs much more rapidly than to others. The observations on recovery and its limits are as yet only fragmentary and further work is necessary before a complete statement can be made.

Some of the data of an experiment with HCl $m/500$ will serve as an example of recovery. Animals 15 mm. and 60 mm. are placed in HCl $m/500$ and at intervals a pair, one of each size, is returned to water. In the young animals all pulsation ceases at once in HCl, in the old it ceases after two minutes. On return to water after 5–10 minutes in HCl pulsation usually reappears

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after 2–3 minutes in both old and young, often slightly earlier in the young. After one hour in HCl pulsation reappears after 10 minutes in the old, and after 12–13 minutes in the young and after 2½ hours in HCl pulsation reappears in the old after 25, in the young after 45 minutes. The longer the exposure the more the young animal falls behind the old in recovery. After 20 minutes in HCl *m*/400 young animals 18–20 mm. did not recover at all while old animals 60 mm. showed slight pulsation after four hours in water. Recovery after KNC is much slower and occurs only in much lower concentrations.

In all concentrations of KOH from *m*/1000 to about *m*/250 a primary acceleration of pulsation rate and increase in strength of contraction occurs, but in concentrations higher than *m*/250 pulsation usually ceases at once or in a few seconds. During this primary acceleration the pulsation rate is often doubled, but since the maximum rate usually persists only a few seconds and is followed by progressive decrease in rate it is difficult to determine whether the degree of primary acceleration differs in a characteristic way with age. It is certain, however, that the retardation following the acceleration occurs more rapidly in young than in old animals, so that pulsation ceases first in the young, as Table IV. indicates.

The opposite primary effects of acid and alkali on the pulsation rate are what might be expected in the light of what we know of their action on living protoplasm in general. In lethal concentrations, however, their action, following the primary effect, is essentially similar and the relations between susceptibility and age are the same for both.

Mitrocoma discoidea.

In this species the jelly is much less firm than in *Æquorea*, and marked shrinkage occurs in the young animals in acids and some other agents. Muscular contraction as distinguished from pulsation is much more frequent even in nature than in *Æquorea*. Frequently contraction brings about a folding of the umbrella with the two halves of the margin approximated or apposed along a straight line, or the margin may become square or very irregular in outline. The contracted condition may persist for several

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minutes, or after very strong stimulation an hour or two before return to the usual form and resumption of pulsation occur. As in *Æquorea*, the ability to contract persists long after the ability to pulsate has disappeared.

In Table V. the chief data obtained with HCl are given. They are essentially similar to those for *Æquorea*.

TABLE V.

Mitrocoma: SUSCEPTIBILITY TO HCl.

Series.		Diameter in Mm.	Conc. HCl.	Duration of Experiment.	No Pulsation after Stimulation.	No Contraction after Stimulation.	Beginning of Shrinkage.	Disintegration.
X.	*A*	18	*m*/1500	114 hrs.	...?	Acclimated: active at end of expt.		
	B	60			114 hrs. −	...?	...?	...?
II.	*A*	18	*m*/1000	55½ hrs.	40 hrs. ±	...?	...?	...?
	B	60			...?	...?	...?	...?
III.	*A*	18	*m*/800	48 hrs.	8½ hrs.	24 hrs. +	24 hrs.	40 hrs. ...?
	B	55			40 hrs. ±	...?	...?	...?
IV.	*A*	15	*m*/700	48 hrs.	At once	24 hrs. ±	30 hrs. ±	40 hrs. ...?
	B	60			15 hrs.	40 hrs. ±	...?	...?
5 V.	*A*	20	*m*/650	34 hrs.	2 hrs.			30 hrs. ...?
	B	45			15 hrs. ±			34 hrs. ...?
VI.	*A*	18	*m*/650	52½ hrs.	26 hrs. ±	24 hrs. ±	22 hrs.	45–52½ hrs.
	B	60			34 hrs. ±	40 hrs. ±	...?	50 hrs. ...?
VII.	*A*	18	*m*/600	51½ hrs.	At once	24 hrs. ±	32 hrs.	45–51½ hrs.
	B	60			26 hrs. ±	...?	...?	...?
VIII.	*A*	18	*m*/500	4¼ hrs.	At once	1 hr. ±	2 hrs.	
	B	60			" "	...?	...?	
IX.	*A*	20	*m*/500	9¾ hrs.	" "	At once		
	B	60			" "	...?		

The first series 5, X. in HCl *m*/1500 shows acclimation as regards the effect on pulsation rate, pulsation persisting longer in the young than in the old. In *Æquorea* also some degree of acclimation usually occurs in this concentration (p. 53). The

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long persistence of pulsation in both members of the pair in Series 5, VI. (HCl *m*/650) as compared with the other series is anomalous and no reason for it is known. Otherwise the data in the table are consistent and show that for the concentrations included, except *m*/1500, the young animal is more susceptible than the old.

Results with KNC show similar relations between age and susceptibility, of course with much lower concentrations. The results of one series on pulsation susceptibility are given in Table VI.

TABLE VI.

Mitrocoma: SUSCEPTIBILITY OF PULSATION TO KNC.

Series.		Diameter in Mm.	Conc. KNC.	Duration of Experiment.	Pulsation.
4. IV.	*A*..	18	*m*/100000	5 hrs.	Pulsation only retarded: rate *A* = ½ rate *B*.
	B..	60			
4. III.	*A*..	18	*m*/50000	5½ hrs.	Very slight, irregular.
	B..	60			Well-marked, regular.
4. I.	*A*..	16	*m*/25000	½ hr.	Ceases in 10–15 min.
	B..	60			" " 15–20 "
4. V.	*A*..	18	*m*/10000	½ hr.	Ceases in 5 min. ±
	B..	55			" " 15 " ±

In the first pair, 4, IV., *m*/100000, the susceptibility is shown, not in the time of cessation of pulsation, but in the retardation of the rate. At the beginning of the experiment rate $A = 2$, rate B approximately, but after five hours rate $A = \frac{1}{2}$ rate B. In the second pair, 4, III., *m*/50000, pulsation in the young animal is almost completely inhibited after 5½ hours, while in the old it is still strong. Here rate A was very much lower than rate B. In the higher concentrations pulsation ceases in a few minutes.

A few experiments with other agents give similar results. In neutral red animals 18–20 mm. long cease to pulsate, undergo shrinkage and begin to disintegrate much earlier than animals 60 mm., although no marked difference in rate or intensity of staining is apparent. In methylene blue 1/30000, increased to

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1/15000 after 24 hours, the animals live for several days with all cellular tissues stained. After 48 hours pulsation rate $A = \frac{1}{2}$ pulsation rate B approximately, where A and B are animals of 18–20 and 50–60 mm. respectively.

Phialidium gregarium.

The youngest animals found were mostly 7–8 mm. in diameter, while the old, sexually mature individuals measured 20–25 mm. Only a few series of susceptibility experiments were made with this species, HCl *m*/1500, *m*/1000, *m*/800, *m*/600, neutral red and methylene blue being used. The susceptibility relations are similar to those observed in the other species. In this rather delicate form shrinkage is a characteristic feature of the action of various agents, the younger animals undergoing shrinkage earlier and to a greater degree than the old, a decrease of half or three fourths in diameter occurring in many cases.

Sarsia rosaria.

In this species the umbrella is very deep, the oral-aboral axis being almost or quite twice the diameter. Depth is therefore a more convenient measure of size than diameter and is commonly used. The sexually mature animals average about 15 mm. in depth of umbrella, but the extremes range from 10–12 mm. on the one hand to 20 mm. on the other. In the smallest and youngest animals found, 6–7 mm. in depth, the gonads had already begun to develop, and although the susceptibility of these forms is characteristically greater than that of the mature individuals the differences in age and in susceptibility in the animals available are not as great as in the other species examined.

In general, results with this species are similar to those with others but in some pairs an individual 12–15 mm. showed a susceptibility equal to or even greater than that of 7–8 mm. animals. Since none of the animals within these limits of size represent very great differences in physiological age, all being near sexual maturity, it is to be expected that occasionally a larger animal will show a higher susceptibility than a smaller.

The agents used were KNC *m*/50000, *m*/10000, *m*/5000, HCl *m*/1000, *m*/600, *m*/500, *m*/400, neutral red and methylene blue,

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and the age differences appeared in all with the few exceptions above noted. One of these exceptions was rather remarkable. In KNC *m*/50000 animals 7–8 mm. usually lose the ability to pulsate, even on stimulation, in 3–10 minutes, animals 15 mm. after 1–2 hours, while in *m*/10000 the times for cessation of pulsation are respectively 1–2 minutes and 3–10 minutes. In one case, however, in KNC *m*/10000 an animal 15 mm. ceased to pulsate in three minutes, while an animal 8 mm. in the same closed container continued normal pulsation during thirty minutes, longer observation being impossible. For some reason this particular animal was practically insusceptible to KNC *m*/10000, a concentration which stops pulsation in the younger individuals of the other species examined in 5 minutes or less and in other younger individuals of *Sarsia* in 1–3 minutes. This extreme exception is merely recorded without any attempt to account for it. The observations on *Sarsia* do indicate a wider range of physiological condition in animals of a given size than in the other species examined, but they afford no clue to interpretation of a condition so extremely exceptional as this.

Conclusion.

The preceding data make it evident that the hydromedusa, like other animals, undergoes a progressive change in physiological condition with advancing development, the differences in behavior and susceptibility being indicators of this change. It has been shown that in other forms a decrease in the rate of oxidations is a characteristic feature of the change and the facts indicate that a similar decrease occurs here, although these forms are not very favorable material for the direct determination of oxygen consumption or CO_2-production. The assumption that the differences in susceptibility are due merely to differences in permeability of surface membranes is refuted by the results obtained with neutral red and methylene blue and by the cases of acclimation. Moreover, there is every reason to believe that the age differences in susceptibility to cyanides are at least more directly associated with the oxidation rate than with permeability.

As regards permeability, however, it is becoming more and more evident to investigators in this field that the condition of

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the membrane is not independent of the metabolic condition of the protoplasm. Permeability to various agents is itself within certain limits an indicator of age or physiological condition of the cell.

The progressive change in behavior, the decrease in pulsation-rate and "spontaneity" indicate very clearly a process of physiological senescence and the data on susceptibility only confirm and extend the facts of observation.

Summary.

Four species of hydromedusæ show evidence of a process of physiological sensecence in the decrease in pulsation-rate and apparent spontaneity and in the decrease in susceptibility to KNC, HCl, KOH and "vital" dyes in various concentrations with advancing development. The criteria of susceptibility are change in rate or cessation of pulsation, loss of contractility, shrinkage of the jelly and disintegration of cellular tissues, and all these criteria agree. The facts justify the conclusion that a decrease in the rate of metabolism, or more specifically of oxidations, is a characteristic feature of this process of senescence.

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BIOLOGICAL BULLETIN

HEREDITY FROM THE PHYSICO-CHEMICAL POINT OF VIEW.[1]

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Heredity, the power of reproducing its like, is a property of all forms of living matter from the lowest to the highest. Broadly speaking, whenever a universal property or mode of action is found, the presumption is that its basis is fundamentally *simple*, for repetition is characteristic of simple rather than of complex conditions and objects in nature: in general, the fewer the variables the more constant the phenomenon. We have therefore to seek for some general or fundamental structural or physico-chemical peculiarity of living things which enables their substance to build up substance of a similar kind. Any form of protoplasm acts as a center of construction of similar forms. At present we are not concerned with the further fact that this reduplication is not perfect, that varieties appear and that diversity has arisen in the course of evolution. This process of divergence is gradual, and even in mutants the differences from their parents are slight compared with the resemblances; the essential fact is that the type is preserved, and that normally the organism appears unable to construct living matter of other than its own kind. The main question is why any species of organism tends so strongly to retain its specific character.

Apparently the most universal property of living matter is its power of proliferation. Out of materials and energy taken from the surroundings it constructs more living matter of the same kind. In other words, the power of *growth* is innate; and if the available nutritive and other materials are sufficient the quantity of the specifically organized and active living substance tends continually to increase. The degree of increase possible under

[1] From the Laboratory of General Physiology, Clark University.

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the conditions may be limited; and in fact most individual organisms and cells have definite limits of size, a balance being eventually reached where metabolic destruction balances construction; nevertheless the construction of new living substance, the essential process underlying growth, continues in all cases throughout life, and when it ceases, life ceases; the constructive process cannot suffer permanent interruption. Even where the organism as a whole seems to have reached its final dimensions there are always special cells or regions which when placed under appropriate conditions still show indefinite proliferative activity. In higher organisms such regions are represented more especially by the germinal epithelium; and a single detached cell from this region exhibits under certain conditions that unique property of definite and orderly proliferation known as development, which leads to the production of an organism similar in its minutest details to that of which the cell originally formed part. This is the chief form which the process of reproduction takes in higher animals, and such a manner of consideration shows that no fundamental distinction can be drawn between growth and reproduction. In many organisms almost any portion of sufficient size which is detached from the whole—whether by some physiologically normal mechanism like fission or as the result of operation—may continue its growth after isolation, redifferentiate, and eventually regain the form and physiological characters of the original stock. Whole plants may thus be reproduced from artificial cuttings, and the same is true of many lower animals (protozoa, hydroids, planarians). In such cases the distinction between growth and reproduction becomes ill-defined, and reproduction appears as essentially a form of discontinuous growth; and in an organism like yeast, where the growing cell-masses may either cohere in chains or fall apart into separate cells—apparently as the result of purely casual conditions—the distinction ceases to have more than a formal significance. If the cell-chain is regarded as the unit organism, increase in its length by budding is a matter of *growth;* if the single cell is so regarded, the same phenomenon becomes an instance of *reproduction.*

It is important for the purpose of the present discussion that the artificiality of this distinction should be recognized at the

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beginning, in order that the nature of the essential problem should be clearly defined. Our aim is to analyze into its simplest physico-chemical terms—so far as this is possible at present—this power of specific construction, of structural and chemical synthesis, which is possessed by all forms of living matter. Reproduction and growth are different manifestations of the same proliferative process. Gametic reproduction is, to be sure, the most highly involved and specialized form of proliferation which we know; yet even here, as also in the case of a simple yeast-cell or bacterium growing in a culture-medium, the process of construction is accomplished through the action of the original germ in incorporating and transforming physically and chemically, in a definite and specific manner, certain materials (food, water, salts, oxygen) taken from the surroundings. The problem of just why the complex and highly organized living system thus built up from a particular species of egg-cell should exhibit its own specific structural, physiological, and chemical peculiarities, and why these should be identical with those of the parent, is one which can be solved in detail only by the special investigation of that particular species. But the fact that any such special development is a prolonged and complex process, involving a progressively increasing differentiation and at length reduplicating the parent form, does not alter its general character as proliferation. The fundamental *general* problem remains the same, whether the process under consideration is the formation of new yeast-cells or bacteria in a culture medium, or the development of a higher animal from a fertilized egg. In both cases material from the surroundings is transformed into living specifically organized substance of a constitution identical with that of the parent organism. And we have to ask whether it is possible, in the present state of our knowledge, to form any clear and consistent conception of the general nature of the physico-chemical conditions under which such a result is accomplished.

A simple concrete instance will define more clearly the nature of the problem to be solved. Consider the case of a single yeast cell introduced into a culture medium. The cell grows and forms buds; these give rise to other buds, and cell-chains are formed from which single cells detach themselves and pass

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through similar transformations; eventually in place of the original cell there are thousands. The non-living material of the culture medium—consisting of water, sugar, ammonium tartrate, and inorganic salts of potassium, calcium, iron, and magnesium—has been transformed into complex and active living protoplasm of a specific chemical and structural organization. Each living cell exhibits the "germ-action" so characteristic of life—*i. e.*, acts as a center of chemical and physical transformation of a definite kind by which more and more yeast protoplasm is formed. We may note here the analogy—to which we shall return—with the process of specific accretion by which a crystal introduced into a super-saturated solution increases its size and becomes a center of deposition of more crystals of the same kind. But the analogy is incomplete; the living organism does not merely change the physical state of the substances which it takes in from the surrounding solution; it also modifies them chemically in the most profound manner, and from the above simple materials it builds up proteins, lipoids, fats, glycogen, and a multiplicity of other substances not present in the culture medium. Further, not only are these substances synthesized, but they are distributed throughout the growing mass of protoplasm in a perfectly definite manner, partly as solid structural material, partly as dissolved or other material serving for energy-production or some form of metabolism.[1] Each one of the specific organized structures thus produced, the yeast cells, reduplicates the physiological activities as well as the structural characters of the parent cell. The result is that the non-living material of the surroundings is transformed or worked over into organized living material of a predetermined type, *i. e.*, the transformation is *specific* and depends upon the nature of the germ originally introduced into the medium. Both growth and heredity are exemplified in their simplest manifestations; and we see again that these terms do not signify two objectively different processes, but merely two aspects of the same process—"growth," the quantitative term, denoting the increase in the total mass of living substance, while "heredity" emphasizes the specificity of the process and its dependence upon the parental character.

[1] *I. e.*, the problem of *differentiation* is inseparable from the problem of growth.

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It is possible to say that each yeast cell *inherits* its properties from the mother cell; but such a phrase serves merely to indicate that the character of the transformation depends upon the character of the germ or proliferating organism itself, rather than upon the character of the material which is transformed under its influence. A mould or bacterium in the same medium effects a totally different kind of transformation, but one equally specific and equally true to the character of the introduced germ.

In general the possession of this automatic property of specific structural and chemical synthesis constitutes the most fundamental distinction between the living and the non-living material systems found in nature. We observe that all organisms and all living cells without exception possess this power; they transform certain inert materials selected from the surroundings into their own characteristically organized and physiologically active living substance. The materials thus incorporated and transformed differ widely in their character and accessibility in different organisms; at the one extreme are green plants living on carbon dioxide, salts, and water; at the other extreme is man in his complex social environment. But this difference of degree does not alter the fundamental identity of kind. Wherever we find life we find exhibited this unique property of specific construction or synthesis, whether the product of the construction is simple or complex.

This property also manifests itself in another and less evident manner. The specific organization of any animal or plant, with the associated physiological activity, not only *originates* in this manner as the product of growth from the parent organism, but once reached, it has to be *maintained.* And this maintenance involves the activity of specific construction in just as full a sense as does growth or development from the germ. During life the organized living substance is continually being destroyed, and must as continually be replaced. This becomes especially evident in certain of the lower animals whenever the food-supply is withdrawn; the quantity of living substance then undergoes progressive reduction, which may proceed until only a small fraction of its original quantity is left; a planarian may thus be reduced by starvation to a quarter of its original length without

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injury, but disorganization and death follow further reduction unless food is restored. A similar loss of living substance through the destructive metabolism inseparable from the life-process takes place in all cells, although its rate and its possible limits vary in the different cases—apparently because of the unequal resistance of different structural elements to regression of this kind; thus in higher animals the loss during starvation is great in voluntary muscle and small in the heart and nervous system. In all cases the specific structural material appears subject to a certain continual breakdown of this kind; even when the non-nitrogenous food-supply is ample for energy-requirements it is found impossible to reduce the nitrogen metabolism—the index of destruction of protein, the specific structure-forming substance —below a certain well-defined minimum. This can only mean that the structural or organized material is subject to continual destruction, and that maintenance involves its continual replacement. This process of replacement is specific, in precisely the same sense in which the growth-process is specific. We must therefore recognize that maintenance involves the activity of specific chemical and structural synthesis in the same sense as does growth. When the construction of organized substance balances destruction there is equilibrium—the condition corresponding to nitrogenous equilibrium in higher animals; any excess of construction leads to growth, of destruction to regression. We see once more that what is essential to continued life is the specific synthetic activity of the protoplasm; when this ceases, life ceases. Claude Bernard expresses this necessary dependence of life upon synthetic or creative processes in the phrase, "life is creation." It is clear that the process of specific creative synthesis which lies at the basis of heredity is inherent in the life-process in all of its forms. The problem of heredity is not a problem to be dealt with by itself; it becomes identical with the most fundamental problem of general physiology, the problem of how living protoplasm is synthesized from non-living matter.[1]

[1] All of this is clearly recognized by Claude Bernard; *cf.* "Leçons sur les phénomènes de la vie," Vol. 2, p. 517, where he summarizes his general view as follows: "The synthetic action by which the organism thus maintains itself [*i. e.*, by a combination of chemical and formative synthesis] is at bottom of the same nature as that by which it repairs itself after it has undergone mutilation, or still further,

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The general physiological problem presented by the phenomena of growth and heredity thus reduces itself to these terms: what are the essential physico-chemical conditions upon which this power of specific construction depends? It seems clear that only a thorough knowledge of the conditions determining the special type of metabolism involved in the process, and especially of its constructive side, can answer such a question. The living organism or cell is primarily a metabolizing system. Growth is the expression or result of a process of metabolism; both the material and the energy required for growth come from outside; within the organism they are transformed in such a manner as to build up an organized system of predetermined kind, the seat of chemical and physical processes which maintain the system and enable it under appropriate conditions to increase in size or to produce other similar systems leading independent life.

In all organisms constructive metabolism involves the synthesis of a multiplicity of new chemical compounds from the food and other materials furnished by the surroundings. The food-materials are typically non-specific in their chemical nature, *i. e.*, they show no relation to the specific character of the organism utilizing them; they are either chemically simple in themselves, or become so during incorporation. In animals, where the organism receives part of its food in highly specific form, as protein, all specificity is invariably lost in the hydrolytic processes of digestion; the material before becoming available for nutrition is reduced to a form in which it can be utilized indifferently by all cells. This non-specificity of the food-materials, as they reach the cells, is in striking contrast with the specificity of the compounds built up from them within the cells. Reduction to a simple or non-specific state is thus the indispensable preliminary to the constructive process. It is therefore highly significant that the chief structural colloids, the proteins, are so readily transformed from the specific to the non-specific state.

by which it grows and reproduces itself. Organic synthesis, generation, regeneration, maintenance, healing of wounds, are different aspects of an identical phenomenon, are varied manifestations of the same agent. . . ." In the Presidential address of J. S. Haldane before the British Association in 1908 (Nature, Vol. 78, p. 555) a similar point of view is expressed, *e. g.* "nutrition itself is only a constant process of reproduction .... Heredity is for biology an axiom and not a problem."

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The universal presence of proteases in cells seems to be the expression of this necessary condition, since in all cells the structural proteins are capable (under certain conditions) of regression and translocation, *i. e.*, of being utilized as food-material elsewhere—the proteins of one region of the cell or organism acting as reserve, so to speak, for construction at other regions. Such a condition is apparently necessary for the normal regulation of cell-structure and activities. For the continuance of normal cell-activity a proper ratio between structural and metabolizing substance must be preserved;[1] hence in every cell the conditions must be present for reducing proteins and other materials to a non-specific and diffusible form, in addition to the conditions for specific synthesis.

By the synthetic activity of the protoplasm these relatively simple substances are united and chemically remodeled so as to form a variety of more complex compounds of which the most individualized and specific are the proteins. The term *specific* is here used as meaning peculiar to the particular organism or cell under consideration, and not occurring elsewhere. It is not a coincidence that living organisms, the most complex systems, in the structural sense, occurring in nature, are also the most complex in the purely chemical sense; and all of the evidence indicates that the structural complexity is the expression or consequence of the chemical complexity.[2] The essential reason for this appears to be that a high degree of chemical specificity or individualization is the necessary prerequisite for structural complexity, and that chemical specificity depends largely upon peculiarities of stereochemical configuration. The number of individualized isomers in the case of any organic compound increases rapidly with increase in the number of asymmetric carbon atoms in the molecule. Hence the proteins, formed of linked amino-acids, most of which are asymmetric compounds, exhibit the possibilities of chemical individualization to a greater degree than any other known class of compounds. It is further significant that proteins which are specifically distinct chemi-

[1] *E. g.*, in a starving protozoön or planarian the normal structure and proportions are preserved, in spite of the decrease in size.

[2] Similarly with structural diversity, whether in the same organism or in different organisms. A corresponding chemical diversity is implied.

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cally, although otherwise closely similar—*e. g.*, hæmoglobins from different species—tend to form crystals, *i. e.*, structural aggregates, which are specifically distinct in their form-characters.[1] A definite relation between the chemical specificity and the structure-forming properties of these colloids is thus indicated. The relation of proteins to organic structure therefore requires special consideration in any theory of heredity.

The proteins form the colloids out of which, together with certain other associated materials, chiefly lipoid, the more permanent or stable, *i. e.*, "structural" portions of most living organisms, and especially the cell-structures, are built up. The relations of these substances to the specific characters of the organism must for this reason be recognized as peculiarly intimate, even though we are not yet in a position to understand the exact relationship between a particular type of structure in an organism and the specific peculiarities of the protein composing that structure. The available evidence indicates, however, that a definite relationship of this kind does exist. We know for example that the presence of foreign proteins is often incompatible with the preservation of normal structure in cells; the cytolytic action of foreign blood-sera, and the formation of specific cytolysins when cell-proteins are used as antigens, show that so fundamental a character as the semi-permeability of the plasma-membrane in a cell is dependent upon the specific peculiarities of its constituent proteins. And in all cell-structures it is probable that a similar relation exists. Such facts indicate clearly that the specific structure of a cell or organism depends upon the chemical specificity of its structural proteins. Now the term specificity, as applied to the individualized character of an organic species, has its morphological and physiological as well as its chemical significance: *i. e.*, each species has its own special form and structure and its own characteristic modes of activity and behavior, as well as its own distinctive and unique chemical composition. But according to the present conception it is the *chemical* specificity which forms the basis of the other two, and

[1] *Cf.* Reichert and Brown: "Differentiation and Specificity of Corresponding Proteins and other Vital Substances in Relation to Biological Classification and Organic Evolution: the Crystallography of Hæmoglobins." Carnegie Institution of Washington, 1909.

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chemical specificity is primarily the property of the proteins. Other biochemical compounds appear to be chemically the same wherever found, but the proteins vary in their specific character from species to species. Moreover, physiologically corresponding or "homologous" proteins are more nearly alike in their chemical and physical characters the more closely related the species are from which they are taken. There is thus a general parallelism between the degree of chemical relationship exhibited by homologous proteins from different organisms, and the degree of biological relationship existing between the species. The indications of this are too various to present in detail in this brief paper, and the evidence has recently been reviewed in an admirable manner by Loeb in his "Organism as a Whole."[1] The specificity which such proteins exhibit when used as antigens, *e. g.*, in the formation of precipitins or specific cytolysins, or in the phenomena of anaphylaxis, shows clearly that the proteins of one species are chemically distinct from the corresponding proteins of even nearly related species, and still more distinct from those of more distant species. Nuttall's well-known work shows that the ability of a given precipitin to react with and precipitate its corresponding protein from another species is a close indication of the degree of blood-relationship between the species under consideration.[2]

Apart from these facts, whose significance in relation to the present problem is now well recognized, there are other evidences of chemical specificity in proteins that offer clearer indications of the nature of the connection between the chemical character of a protein and the character of the structures which it forms in the living cell. The work of Reichert and Brown has shown that hæmoglobin crystals from a given species exhibit form-characters which are definite and specific for the species. This means that when the protein separates from solution in the process of crystallization the molecules, as they unite to form larger crystalline aggregates, by degrees build up structures with definite form-characters—the typical forms of one species exhibiting constant differences from those of other species, even of

[1] *Cf.* Chapter 3: "The Chemical Basis of Genus and Species."

[2] Nuttall, "Blood Immunity and Blood Relationships." Cambridge University Press, 1904.

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the same genus. The growing crystal takes on a definite "species-specific" form, in a manner suggesting a close analogy to the growing germ; this definite form is the expression of the specific properties of the protein molecule, and is presumably dependent upon its special stereochemical configuration. It is well known that molecules of similar stereo-structure tend to segregate in the process of crystallization; thus in the crystallization of a racemic tartrate from its solution one group of crystals is formed exclusively—or at least predominantly—of molecules of the dextro-tartrate, the other of the lævo-tartrate, although in respect to solubility and other physico-chemical properties the two compounds are identical. In such an instance it is quite certain that similarity of stereo-structure is the critical factor determining the union of the dextro-molecules to form a definite crystalline aggregate specifically distinct from that formed by the lævo-molecules.[1]

The physiological properties of the two stereo-isomers are correspondingly unlike; fermentability and related properties (such as general assimilability and pharmacological action) have been shown to differ markedly in a large number of pairs of asymmetric compounds, a clear proof that the activity of living protoplasm is largely conditional upon the specific space-relations of the atoms composing the physiologically active molecules. This is particularly true of compounds entering into metabolism: thus we know that enzyme action is determined by stereo-structure. Perhaps the clearest proof that specific constructive metabolism is similarly determined is furnished by the specific character of the metabolic response following the introduction of protein antigens into the organism—*i. e.*, by the specific character of the anti-bodies produced. These new compounds, evidently synthesized by the living cells, exhibit specific chemical

[1] Thus the introduction of a crystal of the lævo form into a supersaturated solution of racemic tartrate causes the separation of lævo-tartrate alone; similarly dextro-crystals separate out dextro-tartrate (*cf.* Gernez: Comptes rendus, 1866, Vol. 63, p. 843). The recent work of Marc indicates that in general crystallization is preceded by an adsorption; and that crystals, when used as adsorbents, adsorb by preference substances which crystallize in a similar form. *I. e.*, the similarity in the spatial configuration of the molecules is what determines their union to form larger aggregates (*cf.* Marc: Zeitschr. physik. Chem., 1913, Vol. 81, p. 641; also *ibid.*, 1911, Vol. 75, p. 710, and earlier papers there cited).

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properties which are directly determined by the specific properties of the foreign protein introduced. The intimate relationship between the phenomena of immunity and the phenomena of normal assimilation has been pointed out by Ehrlich and others. And since heredity is primarily a matter of assimilation—*i. e.*, construction of the like—it seems clear that the construction of specific protein in the normal processes of growth is the expression of a similar determination of specific constructive processes in the cell by means of the proteins *normally* present. In other words, *the structural proteins already present must determine the production of similar proteins.*

The above tendency of structurally similar compounds to form aggregates when they separate from solution is probably the essential reason why the proteins native to the cell—already forming part of its structure—undergo increase in quantity, with the result that the cell grows. Nothing less than this is to be inferred from the fact that proteins form the basis of specific structures, and that the preservation of the normal characters of any living cell depends upon the continual formation and reformation of these particular compounds. Those proteins which are already laid down as structure within the living cell are thus to be regarded as acting as centres of deposition of further protein identical in composition and configuration. It seems necessary also to conclude that these same structural proteins directly control or guide the actual synthetic process by which more protein of the same kind is built up, presumably by the dehydrolytic condensation of the amino-acids present in the protoplasm. The precise and specific mode of union corresponding to any particular structural protein would then be determined by the specific character of the protein itself. Such a conception would correspond to that of specific catalyzers—the usual manner of conceiving these phenomena at present—the only difference being that the structural protein would itself play the part of catalyzer. Such a process would constitute a form of autocatalysis; the resemblance of growth to an autocatalytic process has in fact been emphasized by Loeb, Robertson, and others.[1] The structural

[1] *Cf.* J. Loeb, *Biochem. Zeitschr.*, 1906, Vol. 2, p. 41. T. B. Robertson, *Arch. f. Entwicklungsmech.*, 1908, Vol. 25, p. 581. Wfg. Ostwald: Roux's *Vorträge und*

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peculiarities of such deposits, and hence the specific morphological characteristics of the cell, would be determined by the specific chemical characteristics of the constituent proteins, in a manner analogous to that by which the special form of a hæmoglobin crystal-aggregate is determined. And the special character of the structure thus laid down would determine the special character of the metabolism, and hence the special type of physiological activity exhibited by the cell. This last conclusion seems inevitable, since the source of this activity is metabolism, which in all living systems is under the control of structure. In other words, the formation of a specific structure in the protoplasmic substratum will necessitate a correspondingly specific type of metabolism, since the nature and rate of the metabolic chemical reactions are controlled by the structural conditions present; the dependent physiological or functional manifestations must therefore also be specific.

We are thus led to conceive certain features of the organic formative process in a somewhat definite manner, which may be summarized briefly as follows: The specific characters of any animal or plant are determined ultimately by the specific characters of its structure-forming proteins. The developing germ or the growing organism synthesizes specific proteins, and these, since they determine the structural and hence the physiological peculiarities of the organism, form the basis of its special character as an organic species. Accordingly one of our most fundamental problems is to determine why the cell builds up proteins of its own specific type. The essential problems of heredity and reproduction center here. As we have seen, heredity is exemplified whenever one yeast-cell or bacterium gives rise to another; also whenever any cell grows and increases its living organized material. This increase in living material is indispensable for the continuance of the species, and for this reason we may characterize growth as the fundamental life-process, and the problem of growth in its most general aspect as identical with the problem of heredity. The factors of growth are the factors of heredity.

Aufsätze, Heft 5, 1908. Chodat made a similar suggestion for plant growth in 1905 (*cf.* D'Arcy Thompson: "Growth and Form," Cambridge Univ. Press, 1917, p. 132).

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Such a conclusion directs our particular attention to the general nature of the conditions controlling growth.

Growth and development may be controlled to a greater or less degree by various artificial means; and much of experimental embryology is concerned with modifying the rate and character of either process. In this way many definite hereditary characters may be profoundly altered, or in some instances their appearance may be altogether suppressed. A simple and instructive instance is described by Loeb.[1] The sea-urchin egg will develop to the gastrula stage in a balanced solution of sodium, potassium, and calcium chloride; if in addition to these salts some sodium carbonate is present, the skeletal spicules may form and a pluteus larva develop, but not otherwise. The skeleton is an inherited character; its formation, however, is dependent upon the presence of sodium carbonate in the surrounding medium, as well as upon the organization of the germ; the necessary carbonate must be furnished to the germ from without, or the specific formative process is unable to take place. Such a result is not difficult to understand. Development, like growth, is a matter of metabolism, and primarily of constructive metabolism; hence it is influenced by any condition that influences metabolism; accordingly the presence or absence of food, oxygen, water, salts, vitamines, hormones, as well as the conditions of temperature, may each and all have determinative relations to the total process. It is significant, however, that the specific characters of the organism, those which, according to the present view, express the chemical specificity of its structural proteins, seem never to be essentially altered by such changes of condition, although their appearance may be prevented or the degree of their development modified. Whatever structural characters appear in development are those characteristic of the species; this statement may be qualified to the degree required to take into consideration the facts of mutation (these suggest that under exceptional conditions new structural proteins may be synthesized); but the essential fact which we wish to express is the tenacity with which the organism preserves its specificity. At least this specificity can be modified, if at all, only by gradual

[1] J. Loeb, *Amer. Journ. Physiol.*, 1900. Vol. 3. p. 441.

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degrees; and any theory of growth or heredity must assign some definite basis for this characteristic conservatism. We have suggested above that this basis is the tendency of similarly constituted compounds to segregate in the formation of aggregates, and in this way to form structures which determine the direction of metabolism. But such an hypothesis explains only the *resemblance* of an organism to its parent; it does not indicate how the proliferative process itself is carried out. It appears, in fact, that two separate groups of problems are involved in the theory of heredity—the one relating to the conditions determining the resemblance to the parent stock, the other relating to the nature of the physiological mechanism by which the living substance (apart from its special nature) increases its quantity or *grows* at the expense of materials taken from the surroundings. Such growth is a physiological activity requiring the expenditure of energy, and it cannot be considered apart from its relation to the other physiological, *i. e.*, functional, activities of the organism. The nature of this latter relation now calls for special consideration.

The general significance of the normal functional activity of the living system as one of the chief factors in the formation of its characteristic structure, or in structure-forming metabolism generally, has been insufficiently regarded by writers on heredity. In general, any normally active tissue maintains itself or grows, while an inactive tissue remains stationary or undergoes regression, even if supplied with an abundance of oxygen and food-material; this last is well shown in a voluntary muscle whose innervation has been interrupted. It is clear that increased functional activity involves an increase of constructive as well as of destructive metabolism; and conversely the subnormal metabolism accompanying inactivity is associated with subnormal construction, which may even fall below destruction, with regression or atrophy as a result. The control which function exercises upon the building-up of living structure is seen perhaps most clearly in higher organisms, and especially in tissues like voluntary muscle, whose activity is intermittent and subject to much variation. In this case the effects of exercise in promoting growth and of disuse in causing regression are

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familiar to all; and essentially similar conditions are found in other tissues and organs. Functional hypertrophy following excessive activity, and regression or atrophy following prolonged inactivity, are both well-known phenomena; for example, compensatory hypertrophy in heart muscle is a frequent result of valvular lesions, and one kidney increases in size if the other is removed. In general it would appear that any physiological function can reach and preserve its highest perfection only through continual repetition; and this very condition implies that decline must follow inactivity if the latter is prolonged beyond a norm. And since every function has some organized structure as its correlate, the same considerations apply to whatever structures are concerned in the function in question. The modifications which the central nervous system undergoes in association with the process of learning afford instances of an essentially similar kind; practise facilitates the repetition of any complex voluntary action, *i. e.*, perfects the structural and other adjustments underlying the function;[1] while any accomplishment, intellectual or other, declines with disuse. These examples may suffice to illustrate the general principle under consideration. It seems clear that the physico-chemical mechanisms—whatever their nature may be—controlling functional activity are in some intimate relation to those determining growth. The above facts seem to imply that both classes of physiological processes are simultaneously and equally under the control of some more general set of conditions characteristic of living substance in general. We shall now consider this possibility in more detail.

Claude Bernard has pointed out how essential it is in any living system—if the system is to continue to exist—that there should be a relation of interdependence between the processes of destruction and of repair, of such a kind that any destructive or dissimilatory process sets in motion automatically the contrary process of repair.[2] All functional activity involves break-

[1] This is the basis of the phenomenon of *memory*. Hering has discussed briefly the relations between memory and heredity in his well-known address on "Memory as a General Function of Organized Matter," Vienna Academy, 1870; English translation by Open Court Publishing Company, Chicago, 1897.

[2] "Leçons sur les phénomènes de la vie," Vol. 1, Chapter 3.

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down of organized material, and apparently some disintegration of cell-structure then always takes place, for protein metabolism is increased—even though slightly under good nutritive conditions—by increased muscular work.[1] Hence for normal regulation of cell-activity it is essential that a compensatory or constructive series of processes should be aroused into action by the same conditions that stimulate or call forth the destructive or energy-yielding activity. "Functional breakdown in living material is itself the precursor and instigator of the renovation accomplished by the formative process, which works silently and obscurely in the interior of the tissue" (*i. e.*, without evident external manifestation, in contrast to the destructive process). "Losses are thus repaired as rapidly as they are caused, and since equilibrium tends to re-establish itself as soon as it is destroyed, the normal composition of the living body is maintained."[2] Bernard also recognizes that this process of restitution

[1] It is now amply demonstrated that increased muscular work in higher animals leads to little or no increase in breakdown of protein (as indicated by N-excretion), provided the non-nitrogenous food-constituents are sufficient in quantity, especially the carbohydrates. If the food contains sufficient protein for maintenance, but carbohydrate and fat are deficient, there may be a considerable increase in N-excretion, but typically not enough to account for the increased energy-production on the basis of oxidation of protein; in this case the surplus of energy comes from the non-nitrogenous reserves of the organism. Carbohydrate is especially effective as a protein-sparer, a fact indicating that in the construction of protein it plays an essential part. It is also the chief source of muscular energy; and the fact that vigorous muscular work, involving active consumption of sugar, is the chief condition for the normal growth of the tissue, shows that the energy required for this growth—*i. e.* for the chemical and structural syntheses involved—is derived from the oxidation of sugar. The possible metabolic changes concerned in this process cannot be considered in an article like the present. But that carbohydrate is essential for the assimilation of amino-acids and other nitrogen compounds in both animals and plants is indicated by a large body of recent and older investigation. Thus for the assimilation of amino-acids by yeast and moulds sugar is indispensible (*cf.* the series of papers by F. Ehrlich, *Biochem. Zeitschr.*, 1906, Vol. 1, p. 8; 1908, Vol. 8, p. 438; 1909, Vol. 18, p. 391; 1911, Vol. 36, p. 477); similarly in higher plants the synthesis of protein from amides in germination requires the presence of carbohydrates (for a brief summary of the facts *cf.* Jost's "Physiology of Plants," p. 175). The work of Loewi, Lüthje and others has shown the great importance of carbohydrates in the synthesis of protein from amino-acids in higher animals; there is also clear evidence that derangement of carbohydrate metabolism (*e. g.*, in pancreatic or other diabetes) interferes very directly with the synthesis of protein (*cf.* Chapter 9 of Cathcart's "Physiology of Protein Metabolism" for a general review of this subject and literature).

[2] "Leçons sur les phénomènes de la vie," Vol. 1, p. 127.

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involves not only a chemical synthesis, but also a morphological synthesis, *i. e.*, a rebuilding of organized structure. The constructive and the destructive processes are inseparable; synthesis is life, whether during rest or activity. Hence the rate of construction must be regarded as ultimately under the same kind of control as the rate of destruction, even though the latter is more obviously subject to modification under the usual conditions of life (as in stimulation, voluntary action, etc.).[1] As instances of the initiation of organic construction by conditions whose primary effect is to stimulate, *i. e.*, to *increase* the energy-yielding dissimilation, Bernard cites the awakening of dormant germs or hibernating animals by rise of temperature or other stimulating condition;[2] the resumption of growth and other processes involving increased assimilation illustrates the constant, though it may be indirect, nature of the interconnection. An apposite present day-illustration of the same phenomenon would be the initiation of development in unfertilized eggs by a temporary cytolytic action. But one does not need to search for instances; the reciprocal interdependence of assimilation and dissimilation is seen everywhere in organisms; how essential this relation is for the preservation of life appears, for example, in the general fact that in all animals increased muscular or other activity hastens the onset of *hunger*, *i. e.*, of the condition necessary for supplying the raw material for construction. The maintenance of a balance between the two kinds of metabolic processes constitutes probably the most fundamental of the various types of organic regulation.

There is no doubt that a general regulatory condition of this kind exists in all organisms; the problem is to determine the essential physico-chemical conditions upon which it depends. We must regard the living system primarily as one in which the synthesis of both chemical substance and organized structure is controlled by functional activity. And during the growth and development of the system, *i. e.*, at the periods when synthetic

[1] Bernard gives direct experimental evidence of the identity of the conditions controlling growth with those controlling stimulation by showing that growth (*e. g.* in seedlings) may be anæsthetized under the same conditions as the different forms of irritability. *Cf.* his chapter on Irritability, Leçons, Vol. 1, p. 267.

[2] *Cf.* Leçons, Vol. 1, Chapter 2, p. 110.

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activity predominates, a similar dependence of formative activity on function must exist. This is the kind of relation emphasized recently by Child, when he describes the developing organism not as being first constructed and then functioning, after the manner of a machine, but as constructing itself *by* functioning.[1] Growth and development are peculiar in that specific construction overbalances destruction, and that the synthesized material, as it accumulates, adopts a definite organization. But this accumulation of structure is itself the expression or result of active functioning, in which energy is freed, just as growth is such an expression in a muscle which has been exercised. Apparently we must conclude that part of this energy is expended in the work of synthesizing and arranging the specific structural material of the organism or cell. We have already seen that this specific material consists essentially of protein. We are thus brought back to the question: what are the conditions under which protein is synthesized in the living cell and deposited as structure?

No very definite or certain answer can be given at present to this question. But we seem to be in a position to rule out certain possibilities, and perhaps to affirm others. First we must note more particularly the significance of the long recognized fact that many vital syntheses require the addition of energy to the synthesized compounds; this is seen, for instance, in the formation of fats from proteins and carbohydrates. Now since such syntheses, where compounds of higher chemical potential are built up from those of lower, take place continually in all cells, it appears highly probable that their conditions are also the main conditions of synthesis in general, and that a subordinate importance is to be attached to the purely enzymatic type of synthesis. Both experience and theory show that the latter is limited to the formation of compounds in which little change of energy accompanies the transformation;[2] hence it is plainly insufficient to meet the normal requirements of constructive metabolism. Some kind of mechanism would seem to be indicated in which energy derived from oxidation or other chemical source is applied

[1] C. M. Child, "Individuality in Organisms." University of Chicago Press, 1915, p. 16.

[2] *Cf.* Höber's "Physikalische Chemie der Zelle und der Gewebe," 1914, p. 677.

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to perform the work of chemical and structural synthesis. Enzymes may facilitate or direct certain kinds of combinations, and in this way they may be important as accessory factors in the constructive process; but the essential controlling factors are evidently of a more active, *i. e.*, work-performing, kind. And since these factors vary in their activity with function, it follows that the conditions controlling the degree and rate of functional activity—*i. e.*, generally speaking, the conditions of stimulation —must at the same time be the conditions controlling the specific constructive processes.[1] It is true that construction does not always run parallel with destruction, the rate of which may often temporarily exceed that of repair; and at times nutritive or other conditions may render complete replacement impossible; or at other times construction may preponderate, as in growth. Nevertheless an interdependence of the kind indicated unquestionably does exist; and apparently we must infer that part of the energy freed in the oxidation (or other energy-yielding decomposition) which performs the work of function is applied, in some manner as yet unknown, to build up the material required for maintenance or further growth.

If we adopt this general hypothesis, we must reject as entirely insufficient the conception of growth as being analogous to a process of crystallization, or as being determined by syntheses of the enzymatic kind; and we are led to look for some other type of process in which the formation and deposition of structural material is controlled by energy set free in chemical change. This process must be capable of variation in rate, of interruption and renewal, and of reversal,—if it is to correspond to such

[1] This is indicated by Bernard s already cited observation that anæsthetics arrest growth-processes reversibly in the same manner as they inhibit stimulation or other forms of functional activity,—a fact suggesting that physico-chemical changes of the same nature control both growth and the response to stimulation. If this is true, it seems probable that the structures primarily concerned in stimulation are also those primarily concerned in construction, *i. e.*, the site or *locus* of both constructive and destructive processes is the same, the two representing reverse phases of the same process. On such a view the idea that special regions of the cell (*e. g.*, the nucleus) are the exclusive seat of syntheses would have to be abandoned. There is, however, much evidence that the nucleus is necessary for the continuance of synthetic processes; possibly it gives rise to certain substances which are required for the maintenance of the structures more immediately concerned in the specific syntheses.

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features of organic growth as the variation of the latter with the conditions (*e. g.*, temperature or the supply of food and oxygen), its dependence on function, and the possibility of regression. Further, it must be a process not necessarily peculiar to living organisms, although apparently taking place under especially favorable conditions in these systems, and it must be able to effect either chemical decompositions or syntheses. All of these peculiarities seem to point to some physico-chemical process of the general nature of electrolysis as underlying the synthetic activity of organisms. In other words the possibility presents itself that electrosynthesis is the chief method of construction in the living system.

The main physiological facts which appear to me to favor this hypothesis are briefly as follows. All functional activity is associated with the formation of electrical circuits between different regions of the cell or organism. The currents of these bioelectric circuits are in many cases sufficiently intense to produce marked physiological effects upon other cells or tissues (stimulation, etc.), and presumably they have similar effects upon other regions of the same cell; the transmission of the effects of local excitation appears in fact to be due to an action of this kind. In general we observe vital functions to be profoundly influenced—accelerated, inhibited, or initiated—by electrical influence; and since function involves specific construction, the constructive process must be subject to similar influence. Experimental data upon the influence of the electric current on growth processes are as yet comparatively few; but galvanotropism is well known in plants, and with properly devised experimentation could probably be shown to be widespread. The control of growth processes by the electric current offers in fact a largely untouched field of investigation, which would probably yield results not only of great theoretical interest but of practical importance as well (*e. g.*, as possibly affording a means of controlling malignant growths, etc.).

But perhaps the clearest indications that the organic formative processes are under the control of electrical conditions are seen in the striking resemblances which certain types of electrolytic deposit show, both in their structural character and in the con-

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ditions influencing their formation, to the structures arising by normal growth in organisms. The two types of phenomena exhibit many close and, as it seems to me, highly significant parallels. Inorganic structures resembling vegetative growths are seen not only in the formation of lead or tin "trees" from metallic zinc immersed in solutions of salts of these metals, but they are shown in an especially striking form in those cases where the local process of electrolysis gives rise to precipitates which form coherent membranes or otherwise exhibit colloidal character. In a recent paper I have described the structure and conditions of formation of such precipitation-structures in some detail, and have discussed the reasons for their resemblance to organic growths.[1] In solutions of potassium ferricyanide, especially those containing egg-albumin or other protective colloid, pieces of metallic iron, zinc, or copper produce characteristic filamentous or quasi-cellular outgrowths, consisting of precipitation-membranes of the ferricyanide of the metal used, which resemble strongly certain definite organic types of growth like mould-hyphæ. These structures grow out into the solution from the anodic regions of the metallic surface; hence their formation may be accelerated, retarded, or suppressed at will by varying the character of the local circuits determining the rate at which the ions of the metal enter solution. A region of (*e. g.*), iron which is actively forming precipitation-filaments will at once cease this action if it is rendered the cathode in another intercepting local circuit,—*e. g.*, by the contact of zinc or other baser metal at a neighboring point; or conversely it may be rendered still more active—*i. e.*, more strongly anodic—by increasing the intensity of the local circuit in which it acts as anode,—*e. g.*, by the adjacent contact of a nobler metal (*e. g.*, platinum) or carbon.[2] It is especially to be noted that the relations between the different electrode-regions of such local circuits are *reciprocal*, as regards the character of the chemical changes there taking place; this is inevitable, since in general the electrochemical processes at any anode are the reverse of those at the cathode. Hence the formation of filaments at one region of the metallic surface appears to have the effect of *inhibiting* their formation at another ad-

[1] BIOLOGICAL BULLETIN, 1917, Vol. 33, p. 135.

[2] *Loc. cit.*, p. 148.

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joining region. A similar reciprocality of influence is especially characteristic of physiological processes, such as excitation and inhibition, *e. g.*, in the central nervous system; and it is also well known to be characteristic of various processes of growth and regeneration in both animals and plants.[1] This is why (for example) cutting off a tubularian head enables an adjoining region of the stem to form a new hydranth; the region where the new growth takes place has been removed (by the operation) from the inhibiting influence of the original hydranth. Similarly a short piece of iron wire which is in contact with a piece of zinc will not form filaments in ferricyanide solution until the zinc is detached or otherwise rendered inactive. Cutting away the zinc thus initiates the development of filaments from the iron;[2] the structure-forming process had previously been inhibited by the activity at the zinc, which on account of its greater tendency to send ions into solution alone forms filaments while the two metals are in contact. To express the matter biologically: the zinc seems actively to appropriate the available structure-forming material (ferricyanide), and in so doing prevents the iron from utilizing this material to form filaments. Similarly the hydranth, with its higher rate of metabolism, acts as the chief structure-forming region in the tubularian, and inhibits structure-formation of the same kind in its vicinity.[3] It corresponds, in this sense, to the anodal metal in the local electrical couple of zinc and iron. The growth-initiating consequences following physiological isolation—to use Child's concise and illuminating expression[4]—may thus be instructively simulated by means of an inorganic model of this kind.

These and similar parallels appear to indicate that *the same type of process* is concerned in the structure-formation in the two kinds of system, otherwise so entirely unlike in character.[5] If

[1] *Loc. cit.*, pp. 156, 163.

[2] *Loc. cit.*, pp. 152 *seq.*

[3] This is an example of the dominance or control of formative processes by those regions having the highest rate of metabolism: *cf.* Child's "Senescence and Rejuvenescence," Chapter 9, p. 210; also "Individuality in Organisms," Chapter 5.

[4] *Loc. cit.*

[5] It is a question whether an electric current passing between any semi-permeable water-insoluble phase and the adjoining aqueous solution can do so otherwise than by a process of ionization (or deionization) at the boundary, *i. e.*, by a process

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this is true, the transmission of the structure-forming or growth-inhibiting influence is in both cases due to the formation of electrical circuits between the influenced and the influencing regions. In their morphological details and in their chemical composition the structures formed are, needless to say, widely different in the living system and in its inorganic model, although in certain peculiarities of physico-chemical constitution—especially the cellular and filamentous character of the formations and the part played in both by semipermeable membranes—the precipitation-structures and the living systems show significant resemblances. A precipitation-structure of a definite chemical composition even shows a certain morphological specificity, *i. e.*, the structures formed from zinc are characteristic in their appearance and different from those formed from iron or copper. We may say that under the influence of the metal the ferricyanide of the solution is transformed into structure of a definite kind. And this structure, once formed, becomes the condition of formation of other similar structure.[1]

To complete the resemblance to a growing plant-filament or other proliferating living system, the structures thus formed ought to be capable, after isolation, of forming more structure of the same kind. This is of course not the case with the precipitation-filaments, taken by themselves, since the connection with the metal is essential; but the difference may be regarded as due mainly to incidental conditions. The growing system is in fact not constituted by the filaments *alone*, but by the combination of filaments, metal and solution; the formative process depends upon the interaction of all three. Something analogous may be said to be true of the growing organism; in a spore placed in a

involving electrolysis. This is what takes place in the passage of a current between a metallic surface and a solution; in this case the addition or abstraction of electric charges to or from substances present at the boundary is what forms the essential condition of the electrolysis there taking place. The cell-surface is similarly water-insoluble, and semi-permeability is characteristic; yet it allows the passage of the electric current, although with a somewhat high and variable resistance. The facts of polar stimulation, polar disintegration, etc., indicate that where the current *enters* the cell-surface it produces different chemical effects from where it *leaves*, just as in electrolysis at metallic surfaces.

[1] *Cf.* the description of the mode of formation of precipitation-filaments, *loc. cit.*, pp. 143–144.

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culture-medium the structure of the germ, its chemical composition, and the chemical composition of the culture-medium, are all equally essential factors in the resulting growth-process. The main difference is that each living cell, as soon as formed, is capable of acting as a similar center of transformation when transferred to another culture-medium; *i. e.*, *all* of the necessary parts of the proliferating system are multiplied equally; and so far we have been unable to produce any artificial systems having such properties. If we were to succeed in doing so, it is probable that such systems would exhibit a much closer resemblance to living organisms than any of the inorganic models hitherto used for comparisons of the above kind.

Obviously these fundamental resemblances between the two types of system under comparison do not preclude infinite differences in the details of structure, chemical composition, and activity; but I am at present insisting upon the resemblances because of the desirability of determining the *class* to which the organic formative processes belong. The characteristic plasticity and responsiveness of living matter undoubtedly depend upon the fundamental features of its physico-chemical constitution. Starting with living material of this peculiar type of self-regulating structure and chemical composition, the developmental process has in the course of time become so evolved and perfected that it now builds up with unfailing regularity the most complex of organisms from the food and other materials furnished to the germ from the surroundings. But the possibility of this development has depended upon certain general pecularities of physico-chemical constitution present from the beginning in the living formative substance itself; and my aim in the present and preceding papers is to indicate what seem to me the most essential of these peculiarities.

The case of higher organisms presents numerous problems of a more special kind, and most investigators in the field of heredity have given their chief attention to these problems. It seems clear that in these organisms other and more special mechanisms of hereditary coördination and control have been superposed upon the elementary physico-chemical mechanism which conditions the fundamental proliferative activity. The fact that in

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higher animals particular cell-structures like chromosomes may become essential regulatory or determinative factors, controlling the detailed character of the developmental proliferation, is in no sense inconsistent with the general point of view here presented. All protoplasmic structures as they originate must influence formative metabolism, as has been so ably pointed out by Child; and there is every evidence that the chromosomes have a peculiarly intimate relation to the distribution of form-determining factors. Recognition of the part played by hormones in development is also consistent with the present view. What we are now attempting, however, is not to define the special mechanisms governing the course of development in the higher metazoa, but to indicate the nature of the more general physico-chemical mechanism, common to all forms of living matter, which forms the basis of its characteristic self-conserving and proliferative properties. Any conception of the essential constitution of living matter must first of all take its constant and fundamental distinguishing peculiarities into account. Once the living material has come into existence, with such properties as these, it may serve as the basis for the development of diversity and complexity of all kinds, as has in fact occurred in the course of evolution.

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APPETITES AND AVERSIONS AS CONSTITUENTS OF INSTINCTS.

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GENERAL ACCOUNT OF APPETITE AND AVERSION.

The overt behavior of adult animals occurs largely in rather definite chains and cycles, and it has been held that these are merely chain reflexes. Many years of study of the behavior of animals—studies especially of the blond ring-dove (*Turtur risorius*) and other pigeons—have convinced me that instinctive behavior does not consist of mere chain reflexes; it involves other factors which it is the purpose of this article to describe. I do not deny that innate chain reflexes constitute a considerable part of the instinctive equipment of doves. Indeed, I think it probable that some of the dove's instincts include an element which is even a tropism as described by Loeb. But with few if any exceptions among the instincts of doves, this reflex action constitutes only a part of each instinct in which it is present. Each instinct involves an element of appetite, or aversion, or both.

An appetite (or appetence, if this term may be used with purely behavioristic meaning), so far as externally observable, is a state of agitation which continues so long as a certain stimulus, which may be called the appeted stimulus, is absent. When the appeted stimulus is at length received it stimulates a consummatory reaction, after which the appetitive behavior ceases and is succeeced by a state of relative rest.

An aversion (example 7, p. 100) is a state of agitation which continues so long as a certain stimulus, referred to as the disturbing stimulus, is present; but which ceases, being replaced by a state of relative rest, when that stimulus has ceased to act on the sense-organs.

The state of agitation, in either appetite or aversion, is exhibited externally by increased muscular tension; by static and

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phasic contractions of many skeletal and dermal muscles, giving rise to bodily attitudes and gestures which are easily recognized signs or "expressions" of appetite or of aversion; by restlessness; by activity, in extreme cases violent activity; and by "varied effort" (Lloyd Morgan, '96, 7, 122, 154; Stout, '07, 261, 267).

In the theoretically simplest case, which I think we may observe in doves to some extent, these states bring about the appeted situation in a simple mechanical manner. The organism is disturbed, actively moving, in one situation, but quiet and inactive in another; hence it tends to move out of the first situation and to remain in the second, obeying essentially the same law as is seen in the physical laboratory when sand or lycopodium powder on a sounding body leaves the antinodes and comes to rest in the nodes.

But pigeons seldom are guided in so simple a manner. Their behavior involves other factors which must be described in connection with appetite and aversion.

An appetite is accompanied by a certain *readiness to act.* When most fully predetermined, this has the form of a chain reflex. But in the case of most supposedly innate chain reflexes, the reactions of the beginning or middle part of the series are not innate, or not completely innate, but must be learned by trial. The end action of the series, the consummatory action, is always innate. One evidence of this is the fact that in the first[1] manifes-

[1] To see the appetitive nature of an instinct, it is necessary in some cases to observe an individual animal carefully during its first performance of the act in question. But the performance may be so quick that the observer is quite unable to analyze it. Analysis may be aided by preventing the animal from attaining the consummatory situation for a time, so that the appetitive phase is prolonged, as it were magnified. My cripple dove (example 5, p. 99) afforded just this aid to analysis. The literature is full of reports of instinctive behavior which might well be further analyzed. Consider for example the case of the young moorhen cited by Lloyd Morgan ('96, 63) which had never previously dived, but on being suddenly frightened by a puppy, dived like a flash. That act was too quick for us to analyze it. But if we could successfully impede the diving of a young moorhen so as to prolong the phases of the act, I think it probable that we should find an appetite for the consummatory situation (that of being under water) and a restless striving until it is attained; and that some details in the series of actions, details which in a normal dive are very sure to be hit upon by accident, are not innately predetermined. When one sees the first performance of an instinctive act take place very quickly and with apparent perfection, this does not prove that there is an innate chain reflex determining every detail of the act.

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tation (also, in some cases, in later performances) of many instincts, the animal begins with an *incipient consummatory action*, although the appeted stimulus, which is the adequate stimulus of that consummatory action, has not yet been received. I speak of an incipient "action" rather than "reaction," because it seems clearly wrong to speak of a "reaction" to a stimulus which has not yet been received. The stimulus in question is obtained only after a course of appetitive, trial-and-error behavior. When at last this stimulus is obtained, the consummatory reaction takes place completely, no longer incipiently. Then the appetitive behavior ceases; in common speech we say the animal is "satisfied."

One may observe all gradations between a true reflex and a mere readiness to act, mere facilitation. Thus, in the dove, a stimulus from food in the crop may cause the parent to vomit the food or to feed it to young: there are all gradations from an immediate crop-reflex, in which the food is vomited upon the ground, through intermediate cases in which the parent is much disturbed by the food in his crop, but appetitively seeks the young and induces them to take the food; to other cases is which the parent is only ready to feed the young if importuned by them; and finally to cases in which the stimulus from the crop does not even cause facilitation, and the parent does not disgorge the food at all, even if importuned by the young.

While an appetite is accompanied by readiness for certain actions, it may be accompanied by a distinct *unreadiness* for certain other actions, and this is an important factor in some forms of behavior. It is altogether probable that this unreadiness is due in some cases to the fact that the activity of certain neurones *inhibits* the activity of certain other neurones. It is now well-known, too, that unreadiness may be due to the condition of the internal secretions. And the mutual exclusion of certain forms of instinctive behavior is inevitable, due to the incompatibility (Washburn, '16) of their motor components.

Unreadiness may be accompanied by aversion, and vice versa; but either of these may occur without the evident presence of the other. An aversion is sometimes accompanied by an innately determined reaction adapted to getting rid of the disturb-

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ing stimulus, or—this point is of special interest—by two alternative reactions which are tried and interchanged repeatedly until the disturbing stimulus is got rid of (see example 7, page 100).

The escape from a disturbing situation or the attainment of an appeted one is accomplished, in case of some instincts, far more surely and more rapidly after one or more experiences. In the first performance of an appetitive action, the bird makes a first trial; if this fails to bring the appeted stimulus he remains agitated and active, and makes a second trial, which differs more or less from the first; if this fails to bring the appeted stimulus he remains still active and makes a third trial; and so on until at last the appeted stimulus is received, the consummatory reaction follows, and then the bird comes to rest. In later experience with the same situation, the modes of behavior which were followed immediately by the appeted stimulus and consummatory reaction are repeated; those which were not so followed tend to drop out.

If a young bird be kept experimentally where it cannot obtain the normal stimulus of a certain consummatory reaction, it may vent that reaction upon an abnormal or inadequate stimulus, and show some satisfaction in doing so; but if the bird be allowed at first, or even later, to obtain the normal stimulus, it will be thereafter very unwilling to accept the abnormal stimulus. That this is true of the sex instinct has been shown in a former article (Craig, '14). It is true also of the appetite for a nest. Thus a female dove which has never had a nest, nor material to build one, lays eggs readily on the floor; but a dove that has had long experience with nests will withhold her egg if no nest is obtainable. The male dove similarly, if he has never had a nest, goes through the brooding behavior on the floor; but an experienced male is unwilling to do so, and shows extreme anxiety to find a nest. These examples illustrate the fact that the bird must *learn* to obtain the adequate stimulus for a complete consummatory reaction, and thus to satisfy its own appetites.

There is often a struggle between two appetites, as when a bird hesitates, and it may hesitate for a long time, between going on the nest to incubate and going away to join the flock, eat, etc. By watching the bird one can predict which line of behavior it

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will follow, for each appetite is distinguished by its own expressive signs (consisting partly of the incipient consummatory action), and one can see which appetite is gaining control of the organism.

These outward expressions of appetite are signs of physiological states which are but little known. Since my own observations have been on external behavior only, I say little about the internal states. They are probably exceedingly complex and numerous and similar to the physiological states which in the human organism are concomitants of appetites,[1] emotions, desires. They doubtless include stimulations from interoceptors and proprioceptors; perhaps automatic action of nerve centers; perhaps readiness or unreadiness of neurones to conduct. It is known that some of the periodic appetites are coincident with profound physiological changes. Thus Gerhartz ('14) found that during the incubation period in the domestic fowl the metabolism of the body as a whole is at a low ebb. In some cases a stimulus from the environment is the immediate excitant of an appetite; especially, stimulation of a distance-receptor may arouse appetite for a contact stimulus, as when the sight of food arouses appetite for the taste of it. But probably in every case appetite is dependent upon physiological factors. And in many cases the rise of appetite is due to internal causes which are highly independent of environmental conditions, and even extremely resistant to environmental interference.

Appetitive behavior in vertebrates is evidently a higher development of what Jennings ('06, p. 309) calls the positive reaction in lower organisms; aversive behavior in vertebrates corresponds to what Jennings (p. 301) calls negative reactions.

The attempt to distinguish between instinct and appetite, as in Baldwin's Dictionary ('01), is not justified by the facts of behavior. Baldwin says : "Appetite is distinguished from instinct in that it shows itself at first in connection with the life of the organism itself, and does not wait for an external stimulus, but appears and craves satisfaction." These characteristics,

[1] Hunger furnishes a typical case of appetitive behavior (Carlson, '16; Ellis '10, 198–199). Carlson makes a distinction between hunger and appetite. The distinction he finds is certainly real, but the use of words is unfortunate, for hunger is clearly one kind of appetite.

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here ascribed to appetite, are the very ones which I have observed in the instinctive behavior of pigeons. The instincts of pigeons satisfy Baldwin's further description of appetite in that each appears first as a "state of vague unrest" involving especially "the organs by which the gratification is to be secured"; and "a complex state of tension of all the motor . . . elements whenever the appetite is aroused either (*a*) by the direct organic condition of need, or (*b*) indirectly through the presence or memory of the object." This last point is illustrated, *e. g.*, by doves learning to drink (example 1, page 97), in whom the sight of the water-dish at a distance aroused the drinking actions by associative memory. I have observed appetitive behavior as Baldwin describes it in nearly all the instinctive activities of doves, and I think that sufficient observation will reveal it in all their instincts.

The most thorough attempt to distinguish instincts from appetites and to show the logical consequences of such distinction, in all the literature to which I have access, is in an old article by Professor Bowen ('46). This article is still worth study, to suggest the conclusions to which one is logically led if he denies that instincts contain any element of appetite. These conclusions, taken almost literally from Bowen, may be summarized as follows: (1) (P. 95) "If the name of instinct be denied to these original and simple preferences [appetites] and aversions, there will appear good reason to doubt whether man is ever governed by instinct, whether all his actions are not reducible to passion, appetite, and reason." (P. 115) The "passions" of man can not be concomitants of instinct. (2) (P. 117) "Instinct is not a free and conscious power of the animal itself. It is, if we may so speak, a foreign agency, which enters not into the individuality of the brute." (P. 118) Instinct "has no effect on the rest of their conduct, which is governed by their own individuality." (3) Bowen contends with logical consistency that if instinct contains no appetitive factor, the ends toward which instincts work, as seen by an observer, are not ends for the agent; that therefore the agent has no power to make the instinctive behavior more effective. In short, instinctive behavior is not susceptible of improvement by intelligence. (4)

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Bowen concludes that the intellect and the "passions" of man are not products of evolution. (5) It may be added that even Bowen, strive as he did to separate appetite from instinct, was compelled to admit that the attempt at such separation leads one into difficulties and disputed cases. In contravention of Bowen's conclusions I contend: (1) That much of human behavior is instinctive. (2) That Bowen's description of instinct as "a foreign agency, which enters not into the individuality" is true of reflex action, such as coughing or sneezing, but is not true of instinctive behavior, which is extremely different from such mere reflexes. (For a fuller statement on this point, see below, page 106. See also Hobhouse, '15, 98–99.) (3) That, of the useful results toward which instincts tend, some, not all, are ends for the agent. For they are the objects of appetites, and the animal strives and learns to attain them. (4) That human conative behavior evolved from the instinctive appetitive behavior of lower animals.

In another article I hope to publish soon a further discussion of the literature.

Examples.

1. The case of doves learning to drink, as described in detail in a former article (Craig, '12), illustrates appetite. The observed appetitive behavior was aroused by stimulation of distance-receptors, such as the sight of the water-dish being brought to the cage, and of the man bringing it; these acted as appetizers. Each dove, as soon as it had learned to associate such stimuli with the drinking situation, responded to these stimuli by making drinking movements (incipient consummatory action) at once without going to the water dish. The first drinking movements failing to bring water, the dove repeated these movements again and again, sometimes walking a few steps, sometimes turning round, until after many trials and many errors it did get its bill into the water, received the stimulus from water in the mouth (appeted stimulus), whereupon the drinking movements (consummatory reaction) were made not incipiently but completely, the water being swallowed, after which the bird rested and appeared satisfied.

2. A good example of appetitive behavior is seen in the way in

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which a young male dove locates a nesting site for the first time. The first thing the observer sees is that the dove, while standing on his perch, spontaneously assumes the nest-calling attitude, his body tilted forward, head down, as if his neck and breast were already touching the hollow of a nest (incipient consummatory action), and in this attitude he sounds the nest-call. But he shows dissatisfaction, as if the bare perch were not a comfortable situation for this nest-dedicating attitude. He shifts about until he finds a corner which more or less fits his body while in the tilted posture; he is seldom satisfied with his first corner, but tries another and another. If now an appropriate nest-box or a ready-made nest is put into his cage, this inexperienced dove does not recognize it as a nest, but sooner or later he tries it, as he has tried all other places, for nest-calling, and in such trial the nest evidently gives him a strong and satisfying stimulation (the appeted stimulus) which no other situation has given him. In the nest his attitude becomes extreme; he abandons himself to an orgy of nest-calling (complete consummatory action), turning now this way and now that in the hollow, palpating the straws with his feet, wings, breast, neck, and beak, and rioting in the wealth of new, luxurious stimuli. He no longer wanders restlessly in search of new nesting situations, but remains satisfied with his present highly stimulating nest.

3. Fetching straws to the nest is apparently due to an appetite for building them into the nest. The dove has an innate tendency to pick up straws, and an innate tendency to build them into the nest (consummatory reaction); but it has apparently no innate tendency to carry a straw to the nest, no innate "chain" of reflexes. When an experienced bird finds a straw he seizes it repeatedly and toys with it, sometimes making movements resembling those by which he would build the straw into the nest. He seems thus to get up an appetite for building the straw in, and when this appetite is sufficiently aroused he flies to the nest, guided by associative memory, and performs the consummatory reaction completely. A young female, no. 70, which I observed picking up a straw for the first (?) time, on her 54th day, showed the lack of a "chain reflex." For she continued toying with the straw an excessively long time, not carrying it at all,

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though she happened to be very near the nest. This was the more remarkable as she had a well-formed habit of going to the nest on all occasions. At length she did go to the nest with her straw, and made well-ordered movements to build it in.

4. The male and the female dove take regular turns in sitting on the eggs. The male is seized by the appetite for brooding about 8 or 9 A. M., and the female about 5 P. M., the state evidently being brought on in each case by physiological causes which are part of the daily physiological rhythm. When either one, *e. g.*, the female, comes to the side of the nest prepared to enter and sit, she already has somewhat the attitude of the sitting bird, the body sunk down on the legs and the feathers fluffed out (incipient consummatory action). If her sitting appetite be thwarted, as by her mate refusing to budge from his position, she shows restlessness and makes intelligent efforts to obtain possession of the nest. When at last her mate yields his place, she steps exultingly into the center of the nest and settles herself on the eggs with many movements indicative of satisfying emotion (complete consummatory reaction).

A broody hen of course illustrates the same principle.

5. It is an interesting fact, exhibited in a variety of instincts, that a young bird may make feints of performing actions which it has never yet performed. Thus the young dove makes feints of flying before it has ever flown. This was illustrated in a peculiarly instructive manner by one of my young doves, no. 46, which developed cripple wings and was unable to fly. When placed in a box with sides 3½ inches high it was just able to jump on the edge. Nevertheless, when its roosting instinct developed, it endeavored strenuously every evening to fly to the perch which was some inches above its head. It looked at the perch and aimed at it with perfect definiteness, opening its wings and making feints of flying. In the evolution of birds, there can be no doubt, flying developed gradually from jumping. The new movements of flying were gradually intercalated into the interval between the initial action, leaping from the ground, and the final action, landing again upon the feet. The young dove to this day shows *first* the incipient end action, aiming at the perch to be alighted on, and only after it has launched itself

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toward this end situation does the "chain" of flight reactions take place.

6. In the pigeons the order of activities culminating in the sexual act is, first display, second billing, third copulation, with numerous details each finding a place in the succession. Yet the sexual tendency is mainfestly present from the beginning of the "chain," and the preliminary steps are directed, with much guidance by experience, toward securing the stimulation required for discharging the sexual reflex. In absence of the normal stimulus to the consummatory reaction, the instinct manifests itself in marked appetitive behavior, and, especially in inexperienced birds (Craig, '14), in those imperfect consummatory reactions known as perversions and auto-erotic phenomena. The behavior of the sexual appetite is now so well known that it may be cited as the type of appetitive behavior; and to readers who are familiar with modern analyses of the sex instinct I may make my whole article clearest by saying that all the appetitive mechanisms I have mentioned, and I believe all the instincts of the dove, behave in the same manner as that of sex, in regard to appetitive manifestations and anticipation of the consummatory reaction.

7. I shall take space to describe only one example of aversion—the so-called jealousy of the male dove, which is manifested especially in the early days of the brood cycle before the eggs are laid. At this time the male has an aversion to seeing his mate in proximity to any other dove. The sight of another dove near his mate is an "original annoyer" (Thorndike, '13, Chap. IX.). If the male sees another dove near his mate, he follows *either of two* courses of action; namely, (*a*) attacking the intruder, with real pugnacity; (*b*) driving his mate, gently, not pugnaciously, away from the intruder. When he has succeeded either in conquering the stranger and getting rid of him, or in driving his mate away from the stranger, so that he has got rid of the disturbing sight of another dove in presence of his mate, his agitation ceases. If we prevent him from being successful with either of these methods, as, by confining the pair of doves in one cage and the third dove in plain sight in a contiguous cage, then he will continue indefinitely to try both methods. If we leave all three

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doves free in one pen, the mated male will try the mettle of the intruder and conquer him if he can; if he fails, he will turn all his energies into an effort to drive his mate away from the intruder. Or if in former experiences he has learned to gage this individual intruder, if he conquered him before he will promptly attack him now, but if defeated by him before he will now choose the alternative of driving his mate away. In sum, the instinctive aversion impels the dove to thoroughly intelligent efforts to get rid of the disturbing situation.

8. In some cases the seeking of a certain situation involves both appetences and aversions in considerable number. Thus, when the day draws to a close, each dove seeks as its roosting-place a perch that is high up, with free space both below it and above it, with no enemies near, with friendly companions by its side, but these companions not too close, not touching (except in certain cases of mate, nest-mate, or parent). The endeavor to achieve this complex situation, to secure the appeted stimuli and to avoid the disturbing ones, keeps the birds busy every evening, often for an hour or more.

Cycles.

Instinctive activity runs in cycles. The type cycle, as it were a composite photograph representing all such cycles, would show four phases as follows.

Phase I.—Absence of a certain stimulus. Physiological state of appetite for that stimulus. Restlessness, varied movements, effort, search. Incipient consummatory action.

Phase II.—Reception of the appeted stimulus. Consummatory reaction in response to that stimulus. State of satisfaction. No restlessness nor search.

Phase III.—Surfeit of the said stimulus, which has now become a disturbing stimulus. State of aversion. Restlessness, trial, effort, directed toward getting rid of the stimulus.

Phase IV.—Freedom from the said stimulus. Physiological state of rest. Inactivity of the tendencies which were active in Phases I., II., III.

Some forms of behavior show all four phases clearly. The following are examples.

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Sex.—(Phase I.) The dove, either the male or the female, shows sexual appetite and invites the mate to sexual activity. Gradually they lead up to (Phase II.) the consummatory sexual act. (Phase III.) After the sexual act, in some cases one bird shows marked aversion, *e. g.*, by striking at the mate. Either the male or the female may show aversion. In some species, signs of aversion after the sexual act seem to be a normal and regular occurrence. In other species they are shown only by a bird whose mate, having failed of satisfaction, invites to further sexual activity. (Phase IV.) The pair usually become sexually indifferent for a considerable time after each copulation.

Brooding.—(Phase I.) The dove shows the brooding appetence, goes to the nest, and, if need be, struggles to obtain possession of it. (Phase II.) It sits throughout its customary perood, during which it often resists efforts of the mate to relieve it. (Phase III.) At the end of this period, in contrast, it comes off at a slight sign from the mate, runs about, flaps its wings, and thus shows its joy in being off. This may be interpreted as a sort of mild aversion for the nest. (Phase IV.) It goes away and becomes temporarily indifferent to the nest.

In other cases, one or other of the phases is not clearly present, so that there are various sorts of incomplete cycles, such as the following.

(*a*) When the bird shows appetitive behavior but fails to obtain the appeted stimulus, the appetite sometimes disappears, due to fatigue or to drainage of energy into other channels; in which case, Phase II. is not attained.

But many instinctive appetites are so persistent that if they do not attain the normal appeted stimulus they make connection with some abnormal stimulus (see page 94); to this the consummatory reaction takes place, the tension of the appetite is relieved, its energy discharged, and the organism shows satisfaction. This is of course *compensation*, in the sense in which that word is used in psychiatry. But the abnormal stimulus is usually inadequate or incomplete, the relief or discharge is imperfect, the satisfaction is marred by the fact that some of the constituent elements of the appetite, failing to receive their appeted stimuli, are still in Phase I. and abnormally active, while at the same time other elements have already reached Phase III., aversion.

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(*b*) Some forms of behavior consist of appetite and satisfaction which are not, in ordinary cases, followed by any distinct aversion. For example, the drinking cycle shows clearly: (Phase I.) appetite for water; (Phase II.) the drinking reaction, with expression of satisfaction; (Phase IV.) indifference. The dove when it finishes drinking shows no distinct sign of aversion (Phase III.) except withdrawing the bill from the water. But if the observer takes this dove then gently in the hand and re-submerges its bill in the water, it shows marked aversion, struggling to withdraw the bill and to shake the water out of it.

(*c*) On the other hand it may seem that there are some forms of behavior, *e. g.*, fear, in which Phases I. and II. are lacking; that there is no appetite for the fear stimuli and no satisfaction in them; that when the slightest of these stimuli is received it at once arouses (Phase III.) aversive behavior. Yet it is an interesting fact that even in these cases a slight degree of appetite and satisfaction may be present. Children seek and enjoy a little fear. A dove, when it hears the alarm cry from other doves, at once endeavors to see the alarming object. Even pain is (in man) to some degree, sought and enjoyed.

In actual life the cycles and phases of cycles are multiplied and overlapped in very complex ways.

For example, when a certain satisfaction has been attained, this, instead of leading at once to a state of surfeit and aversion, may lead to further appetite, which leads to a second satisfaction, and so on. Thus Phase I. and Phase II. continue to alternate, constituting a "circular reaction" (Baldwin). I have seen a pair of house sparrows copulate thirteen times in immediate succession, and know by the sound of their voices that I did not see the beginning of the series. In many cases such circular reaction serves to rouse the organism to a high state of appetite and readiness for action.

Smaller cycles are superposed upon larger ones. For example, when a female bird is building a nest, so long as she is in the nest she is in a certain nest-building attitude, a high state of satisfaction, which constitutes the consummatory reaction (Phase II.) of a large cycle. But each time she reaches for a straw, seizes it, and tucks it into the nest, she exhibits thus a little cycle containing a little appetence followed by its own satisfaction.

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The time occupied by a cycle varies extremely, from cycles measured in seconds to those that occupy a year or even longer. The relative duration of the phases also is extremely variable. In some cases the appeted situation is attained without delay, and Phase I. thus passes so rapidly as to be overlooked by the observer. In other cases the bird strives hard to overcome great obstacles which stand in the way of the attainment of the appeted stimulus, consequently Phase I. is of long duration. Phase II. may last, in the case of drinking, about one second; in the case of incubation, about three weeks.

It should be stated, too, that the phases are not sharply separated; each passes more or less gradually into the next. Thus, from Phase IV. of one cycle in a series to Phase I. of the succeeding cycle, there is often a gradual rise of appetite; active search for satisfaction does not commence until a certain intensity of appetite is attained. This is what is known in pedagogical literature as "warming up." This gradual rise of the energy of appetite is followed (Phase II.–III., or II.–IV.) by its sudden or gradual discharge. This rise and discharge are named by Ellis ('03), in the case of the sex instinct, "tumescence" and "detumescence." They are important phases in the psychology of art, in which sphere they are named by Hirn ('00) "enhancement" and "relief." The discharge (Phase II.) is also exemplified in "catharsis" in art and in psychiatry.

The cycles in the behavior of birds are fundamentally the same phenomenon as the cycles in human behavior. Human cycles are enriched by an intelligence far surpassing that of doves, but this is a difference of degree only. If the dove's cycles are determined largely by instinct, habit, physiological conditions, and not intelligence, so are some human cycles, as those of sleeping, eating, drinking, sex. F. H. Herrick ('10, 83) emphasizes the fact that a bird may scamp one cycle in order to begin another. Thus, birds may abandon young which are not yet weaned, because their appetence for a new brood has set in. But the same principle works, though not quite so crudely, in human life; as in the case of a mother who grows indifferent or even somewhat hostile toward her older children each time a new child is born. Herrick emphasizes also the fact that when anything disturbs

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the bird in the progress of a cycle, she very often gives up that cycle and begins a new one. Thus, a cedarbird who has just completed her nest one day finds a man examining it; she forthwith abandons that nest and begins to build another. But, again, the same phenomenon appears in human behavior. A man begins to build a house; when he has progressed far with the building he meets some horrible experience in it which "turns him against" it, and nothing will induce him to proceed with that house; he abandons it and begins to build elsewhere. The cedarbird has had a, to her, horrible experience which has turned her against her nest; that nest has lost its *value* for her; the sight of it now, instead of arousing her appetence, arouses aversion.

C. J. Herrick ('15, p. 61) says that many of these cyclical activities of birds are "simply complex chain reflexes." The reason he gives for this statement is that "each step in the cycle is a necessary antecedent to the next, and if the series is interrupted it is often necessary for the birds to go back to the beginning of the cycle. They cannot make an intelligent adjustment midway of the series." But all this, in some degree, is true of the behavior of human beings toward their mates, their nests, and their young. This has been illustrated in the preceding paragraph, and a few illustrations are here added. As to mates: When the cordial relation between a husband and wife is, by some mischance, broken, the pair may make an "intelligent adjustment" if the difficulty is not too great. But birds also make such adjustments constantly, when the difficulty is not too great. And with human beings, as with birds, the difficulty may be insurmountable; in which case, the husband and wife separate for a week, a month, or a year, after which period of rest (Phase IV.), they can commence a new cycle with Phase I., courtship. As to their nests: The fact of homesickness proves that the behavior of a human being toward his or her home runs in a series which conforms to Herrick's statements. As to behavior toward the young: The inability of human parents to make "an intelligent adjustment midway of the series" is shown by the fact that they cannot arouse the fullest degree of parental behavior toward an adopted child unless they adopt the child in its infancy. These facts do not prove that the human behavior

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in question consists of mere chain reflexes. Neither do the similar facts as to avian cycles prove that the avian behavior consists of mere chain reflexes.

The birds in their cycles exhibit attention (using this and all the following terms in a strictly behavioristic sense), intelligence, memory, intensely emotional behavior, conflict of tendencies, hesitation, deliberation (of course an elementary sort of deliberation), rise, maintenance, and decline of appetences, behavior conformable to certain laws of valuation. All these forms of behavior function in bringing about the consummatory situations of the cycles. Thus the instinctive behavior of birds, so far from consisting of mere chain-reflexes, and having no relation to "individuality" (Bowen, vide ut supra, p. 97), is in reality very highly integrated, and is the very core of the bird's individuality.

All human behavior runs in cycles which are of the same fundamental character as the cycles of avian behavior. These appear in consciousness as cycles of attention, of feeling, and of valuation.

This description is true not only of our behavior toward objects specifically sought by instinct, such as food, mate, and young, but also of our behavior toward the objects of our highest and most sophisticated impulses. Consider, for example, the course of a music-lover's feelings and attention in the case of a symphony concert. Before the concert, if his internal state is favorable (Phase I.), he is all eagerness, desire, interest. He goes to the concert-hall, chooses a good seat for hearing, and in every way shows appetitive behavior. (Phase II.) The music begins, he pays close attention, and feels satisfaction. (Phase III.) If the concert continues too long, he is surfeited, his pleasure diminishes, he even feels some unpleasantness, and his attention turns away, which is of course a form of aversion. (Phase IV.) When the music at length ceases he feels restfulness, relief, and his attention goes elsewhere. This cycle of the whole concert is overlaid by a complex system of epicycles, each extending through one symphony, one movement, or a smaller division, down to the measure and the beat. This is only one illustration of the fact that the entire behavior of the human being is, like that of the bird, a vast system of cycles and epicycles, the longest cycle extending through life, the shortest ones being measured

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in seconds. This view helps us to understand the laws of attention; for example, the law that attention cannot be held continuously upon a faint, simple stimulus. For as soon as such a stimulus is brought to maximum clearness, which constitutes the consummatory situation, the appetite for it is quickly discharged and its cycle comes to an end. This familiar fact shows that we, like the birds, are but little able to alter the course of our behavior cycles.

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FURTHER STUDIES ON THE GEOTROPISM OF PARAMECIUM CAUDATUM.[1]

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(With two figures.)

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I. INTRODUCTORY.

As evidence of the existence of proper mechanical conditions in protoplasm as a basis for the "statocyst theory" of geotropism in *Paramecium*, Lyon states: "The animals were strongly centrifuged for several minutes in the hæmatocrit attachment. Microscopic examination showed that certian dark granules originally distributed were now aggregated in one end, usually the anterior. It is thus seen that differences in specific gravity exist in the protoplasm of this animal."[2] In previous experiments in which he attempted to test Lyon's results, the writer "could obtain nothing definite"; and the tentative suggestion was made that "immediately after centrifuging a capillary tube containing *Paramecia*, and in which the latter can not turn around, we may stain them with some dyes and determine the effects

[1] From the Physiology Laboratory of the University of Minnesota.

[2] Lyon, E. P., 1905, "On the Theory of Geotropism in *Paramecium*, *Am. Jour. Physiol.*, Vol. 14, p. 430.

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of the centrifugal force upon the protoplasmic materials of the organisms."[1]

While the writer was conducting experiments with a vital stain method in the physiological laboratory of the University of Minnesota, and after he had obtained some valuable results, he learned that McClendon had already published satisfactory results in 1909.[2] The writer found that McClendon's method showed better results than those obtained with vital stains and has, therefore, adopted that method for this investigation, although not able to agree with all of McClendon's interpretations.

This paper attempts to show that *Paramecium caudatum* contains protoplasmic substances of different specific gravities, and subjects to further experimental examination the various phenomena of geotropism in the animal, described by various investigators. The experimental work was done during the academic year of 1913–1914, while the writer was holding a Shevlin Fellowship in the medical school of the University of Minnesota.

II. Material.

A dense culture raised in hay infusion from a single individual was used.

III. Experimental.

1. *The Specific Gravity of Paramecium caudatum.*

As to the difference of the specific gravity of the *Paramecium* between Lyon's 1.048 or 1.049 and the writer's 1.037 or 1.037 ± 0.003, the latter suggested[3] a possible source of error in Lyon's experiments. The writer this time examined the subject more carefully.

He prepared a gum-arabic solution using distilled water, as in the case of his previous experiments. He dialyzed the neutral solution through parchment paper. The specific gravity of the solution so prepared was determined by means of a U-shaped picnometer, as 1.0426.

[1] Kanda, Sakyo, 1914, "On the Geotropism of *Paramecium* and *Spirostonum*," Biol. Bull., Vol. 26, p. 22.

[2] McClendon, J. F., 1909, "Protozoan Studies," *Jour. Ex. Zool.*, Vol. 6.

[3] Kanda, Sakyo, *loc. cit.*

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Then the writer made many small tubes for hæmatocrit attachment, of about 4.7 cm. long and 0.3 cm. in diameter, inside measure. One end of each tube was sealed. One of these tubes was filled with the gum-arabic solution mentioned above and the number of drops which were put in the tube was counted. Now, to the number of the drops of the solution, one drop of water was added. Thus the difference of the specific gravity of the solution before and after the addition of one drop of the latter to the number of drops of the former could be estimated. The writer thus found that the addition of only one small drop of water to the gum-arabic solution of the known specific gravity, that is, 1.0426, lowered the latter to 1.0415. And when another drop of water was added to this, it became about 1.0404. The addition of one drop of water, therefore, lowered the specific gravity of gum-arabic solution about 0.0011.

A pair of tubes was thus prepared with a definite number of drops of the gum-arabic solution of the specific gravity 1.0415. This pair was centrifuged as usual. Then on the top of the solution of one tube, one drop, and on the other, two drops of water containing dense *Paramecia* were added. The tubes were again centrifuged for two minutes with a speed of about 7,300 revolutions per minute. The results were determined by means of a magnifying glass immediately after the centrifugalization. All the procedure was the same as the writer described in his previous paper. Thus the nearest possible density of the animal was obtained:

TABLE I.

I.	Tube 1. Dens. of gum-arab. sol. 1.0404. All stay at upper part. Tube 2. Dens. of gum-arab. sol. 1.0393. Majority stay near middle.
II.	Tube 1. Dens. of gum-arab. sol. 1.0382. Many stay at middle. Tube 2. Dens. of gum-arab. sol. 1.0371. A few go to bottom.

This was carefully tried with different cultures and no practical difference was found. The writer, therefore, concludes that the specific gravity of *Paramecium caudatum* is between 1.0382 and 1.0393.

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In comparison with the writer's previous results, that is, 1.037 or 1.037 ± 0.003, the difference is not great. In his previous experiments, however, he was not critical enough to test the lowering of the specific gravity of the gum-arabic solution after one or two drops of water containing *Paramecia* were added to the tubes. The writer, therefore, considers that the present result is more correct than the previous one.

A more accurate estimation could possibly be made by having the animals before adding them to the centrifuge tube in gum solution of a density only a little less than that of the animals. The lowering of specific gravity by mixture would thus be lessened. Nevertheless, the writer believes that the assumption of 1.039 as the specific gravity of *Paramecium caudatum* is close enough for biological purposes.

2. *The Effects of Centrifugal Force on the Protoplasm of Paramecium caudatum.*

The specific gravity of the animal being known as about 1.039, a gum-arabic solution of higher specific gravity, that is, about 1.1, was prepared. If *Paramecia* are strongly centrifuged in such a solution, they all should be suspended in the solution. And the heavier end of the animals should, also, be passively thrown away from the axis of the centrifuge. Now the writer put a certain number of drops of the gum-arabic solution in a pair of hæmatocrit tubes already described, and on the top of each, one drop of water containing dense *Paramecia* was added. The solution and the water in the tubes were well mixed by means of a fine glass needle. The tubes were centrifuged for fifteen minutes at a velocity of 108.3 revolutions per second and with radius of 2.5 cm. to 7 cm.

The paramecia with some of the gum-arabic solution, after being centrifuged, were sucked out in a capillary tube and were killed in 1 per cent. chromic acid, being left in the solution for about one minute. Then they were stained by McClendon's method, "in Biondi's methyl green, orange G and acid fuchsin mixture with a little less fuchsin and of about one fourth saturated strength"[1] for about four minutes, dehydrated and mounted.

[1] McClendon, J. F., *loc. cit.*

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The microscopic examination of the preparations showed that the darker and heavier substances and crystal-like materials lay in the extreme anterior end of the animal which was thrown away from the axis of the centrifuge. Next to these came the micro-

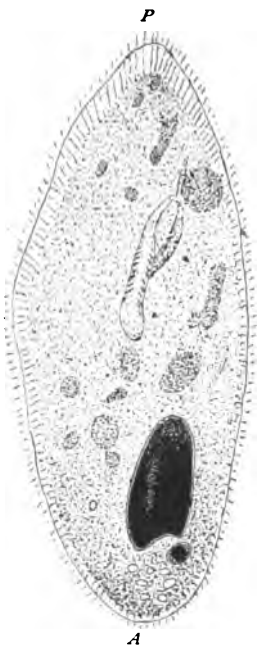

FIG. 1.

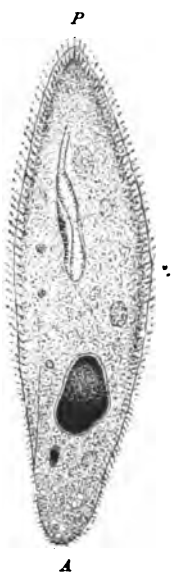

FIG. 2.

FIGS. 1 and 2. Camera drawings of *Paramecium caudatum*. *A*, anterior end; *P*, posterior end.

nucleus and then the macro-nucleus. The chromatin which was stained green, seemed to have been precipitating to the outer nuclear wall, since this part of the nucleus was stained much darker than the inner, as Figs. 1 and 2 have shown. The "plasmosomes" and cell granules were stained orange, but the cilia did not show very distinctly. The writer thus found in confirmation of Lyon's and McClendon's results that *Paramecium* contains protoplasmic materials of differing specific gravities. Figs. 1 and 2 show specimens drawn with the aid of a camera attachment.

That the anterior end of the animal, when centrifuged, was thrown away from the axis of the centrifuge presumably on ac-

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count of its heavier weight, was originally demonstrated by Lyon. This was the strongest in his experiments to cast aside the "mechanical theory." The writer supported him by furnishing results obtained by improved methods to meet Harper's criticism.[1] Harper insisted that in strong centrifugalization, "the same effect is produced at the outset as by mechanical agitation, *i. e.*, the reaction changes to positive." In other words Harper claims that the head is turned outward by a positive response on the part of the organism, and not passively, as claimed by Lyon. The writer showed in his previous paper that Harper could not be right.

However, McClendon thinks that "the geotropic reaction" of the animal "may be strong enough to turn the anterior end in the opposite direction" toward the axis of the centrifuge against the centrifugal force. This needs a serious examination. If the anterior end of the animal is considered "heavier" than the posterior, as Lyon and Kanda hold, it is hardly conceivable that such a small animal as *Paramecium* could orient itself with the heavier end toward the axis of the centrifuge against the force McClendon used. On the other hand, if the posterior end is regarded "heavier" than the anterior, as Verworn[2] and Harper hold, one meets the same difficulty to conceive how the animal can orient itself with the "lighter" anterior end away from the axis of the centrifuge against the much greater force used by Lyon and the writer. McClendon admits that "this is usually the initial orientation," but seems to think the animals may occasionally turn around later by an active reaction. The writer must confess that he cannot conceive its possibility. He found one individual among several dozen examined which was thrown with the posterior end away from the axis of the centrifuge. He considers, however, that this was a mere accident, the animal being oriented in that direction at the beginning of centrifugalization and forced into the capillary before passive turning could occur. McClendon's case was also probably exceptional and moreover had been centrifuged twenty-four hours, during which

[1] Harper, E. H., 1911, "The Geotropism of *Paramecium*," *Jour. Morph.*, Vol. 22, p. 998.

[2] Verworn, Max, 1889, "Psycho-Physiol. Protisten-Studien," Jena: Gustav Fischer, p. 121.

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admitted changes in specific gravity had occurred, due to loss of water. If this loss were chiefly from the posterior part, the changed orientation would be explained as a passive instead of an active process. The writer, therefore, holds still that these animals, when strongly centrifuged, are *passively* thrown with their anterior ends away from the axis of the centrifuge. Therefore, the anterior end is heavier. The negatively geotropic orientation in the normal animals is, therefore, an active process and the mechanical theory is not correct.

McClendon "found the time elapsing before the return of the negatively geotropic reaction to roughly correspond to the time required for the return of the nuclei to their normal position." The writer also found that this was the case. According to McClendon, "this might indicate that nuclei in normal position acted as statoliths." It is hard to decide for or against this for at the time of "the return of the nuclei to their normal position," other heavy substances, "phosphate crystals," for instance, which were precipitated in the extreme anterior end of the animal seem, also, to distribute in their original position. In other words, about at the time of return of the nuclei to their normal position, all the protoplasmic substances of the animal, which were disturbed by the centrifugal force from their original distribution, recover from the disturbance. The result is that the animal resumes the negatively geotropic reaction. Such consideration may suggest that the whole organism in normal conditions acts as a "statecyst," and all the heavy substances as "statoliths," not merely the nuclei. Here lies the significance of Lyon's conception that "for internal stimulation the relation of the parts of the cell to each other must be changed in some way by gravity. Stresses or pulls which occur when the organism is in one position with respect to the vertical, must be changed in another position." For the present we cannot decide in favor of any particular organ or constituent of the cell as the basis of the reaction.

3. Does temperature reverse the negative geotropism of *Paramecium caudatum?*

From his observations on the effects of raising and lowering temperature on the geotropism of this animal, Sosnowski con-

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cludes: "Es hat sich dabei herausgestellt, dass durch Einwirkung höher Temperaturen . . . bei vielen Culturen vorübergehend positiver Geotropismus hervorgerufen werden kann." And "durch die Temperaturerniederingung (bis + 2°) konnte ich auch bei den sehr dazu geeigneten Aquarien keinen positiven Geotropismus hervorufen."[1] On the contrary, Moore[2] states: "Several tubes were left for three hours in a thermostat at a temperature of 26°–28° C. The *Paramecia* collected at the top in dense clusters." And "tube *B* was placed in a larger tube filled with water and surrounded by a mixture of ice and salt, the temperature being kept as nearly as possible at 1° C. In ten minutes the *Paramecia* in tube *B* were massed at the bottom, and two hours later were still massed there."

Sosnowski and Moore, however, gave no consideration to the phenomena of the convection currents which would invariably occur, when water was cooled or heated. That water currents would produce effects on the geotropism of the animal was mentioned by the writer in his previous paper. The animals are rheotropic. Moreover, the animals being so small cannot resist any strong current. Consequently they would be passively carried by the current, when it was strong. There was, therefore, needed a special device to minimize these effects as much as possible.

Dewar vacuum tubes served to some extent for this purpose. The tubes were 3.2 cm. in diameter and 27 cm. long inside measure. Three tubes, one for control and the others for experiments, cold and warm, were used for one series of experiments. It was noted that warm water of 30° or 35° C., which was placed in one of the tubes, with a rubber stopper, cooled off about 2° C. after one hour, and water of 1° to 4° C. in the other became warmer 2° C. in about half an hour. Temperature of the control, that was room temperature, was about 20° C. and fairly constant. The dense culture of animals that were thoroughly washed was cooled or warmed as desired. And a few drops of the culture so

[1] Sosnowski, J., 1899, "Untersuchungen über die Veränderungen der Geotropismus bei *Paramecium aurelia*," *Bull. Intern. d. l'Acad. d. Sci. d. Cracovie*, S. 134.

[2] Moore, Anne, 1903, "Some Facts concerning the Geotropic Gathering of *Paramecium*," *Am. Jour. Physiol.*, Vol. 9, pp. 239 and 240.

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prepared were transferred by means of a pipette into the Dewar tube with special care to make the least current. The tube was closed with a rubber stopper. The results based on many experiments are given in Table I.

TABLE I.

EFFECTS OF TEMPERATURE (?).

Time.	Cold.	Control.	Warm.
	1°–2° C	20° C.	20°–30° C.
Immediately after.	Very sluggish and hardly active.	Majority swim downward.	Abnormally active and majority swim down.
5–10 m. later.....	Not very active and scattered all over.	Many at the lower part.	Many at the lower part.
20–30 m. later....	A little more at the lower part and becoming somewhat active. The temp. about 4° C.	Many at the upper part.	A little more at the lower part. The temp. about 29° C.

Cooling water to 3°, 4°, or 5° C. and warming to 35° C. were also tried. At a temperature 5° C., the animals were somewhat active, and showed a tendency to swim downward. Another series of experiments was also carried out in an incubator and in an ice box. Each experiment lasted three days. The temperature in the incubator ranged from 30° C. to 34° C. In one series of the experiments, the animals so treated in the incubator were "positively geotropic"? That is to say, a majority of the animals always staid at the bottom of the tube, as Sosnowski observed. But in others, the results were very irregular and variable. Experiments in the ice box were not satisfactory, because it was very hard to keep the temperature constantly low.

The writer found that the animals always swam downward, whenever transferred, no matter how carefully it was done. This occurred invariably even in water of the same temperature as the culture, when the animals were transferred into it. It seems to the writer, therefore, to be possible that this reaction was mistaken as reversibility of geotropism of the animals by temperature. That the animals swim downward, *i. e.*, their negative geotropism is reversed, whenever transferred, would suggest

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that mechanical agitation of transferring may be responsible for it. Sosnowski also suggested this factor in one case, "die Temperaturerhöhung und Erschütterung sich zu summieren scheinen" The reversibility of the negative geotropism of the animals by mechanical "shock" or agitation is a well-known fact. The water current may also help it to some extent.

From these results the writer concludes that so-called reversibility of the negative geotropism of *Paramecium* by temperature is extremely doubtful. Especially in such cold water as near 1° C., the animal is almost, if not thoroughly anesthetized. In consequence, it sinks by its weight, though Moore thinks it is "reversed."

4. Do chemicals reverse the negative geotropism of *Paramecium caudatum?*

According to Sosnowski, "durch Zusatz geringerer Mengen von Säuren oder Alkalien (1 bis 3 c.c. Salzsäure oder Natronlauge von 0.5 per cent. auf 20 c.c. Culturflüssigkeit) kann man verubergehenden positiven Geotropismus hervorufen." Moore also states that "in $n/16$ NaCl, they went to the bottom almost immediately, but shortly after died." In a solution of calcium chloride isosmotic with $n/32$, "they went immediately to the bottom."

The writer attempted to test these statements of Sosnowski and Moore. First of all, he thoroughly washed the animals in boiled then cooled tap water. Chemical solutions from the strongest to the weakest were carefully prepared using boiled tap water. A few drops of culture containing dense *Paramecia* were transferred with special care to avoid making water currents as much as possible. The results given in Table II. are to be considered as the best parts of many series of experiments that were quite extensively carried out.

As the table shows, the majority of the animals always swam downward, *i. e.*, were positively geotropic, whenever transferred into any solution as well as in the control, *i. e.*, transfer into culture water. Among the substances used, perhaps, $CaCl_2$ solution "affected" the animals most. In this respect, Moore's observation seems to be confirmed, but it needs further consideration. In any solution, the animals that swam to the bottom did

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not stay there very long. They resumed their negative geotropism within one hour. Sosnowski already observed the same and stated that "wenn sich darauf die Tiere wieder an der Oberfläche sammeln, kann man durch Zusatz einer neunen Quantitat Säure resp Alkali wieder die Ansammlung am Boden versanlassen." This statement is true. But it should be remembered that the same is also true when some tap water is added. It seems to the

TABLE II.

EFFECTS OF CHEMICALS.

	Room Temp. 20° C. Time.					
	Immediately after Treatment.	5 Minutes after Treatment.	10 Minutes after Treatment.	20 Minutes after Treatment.	30 Minutes after Treatment.	60 Minutes after Treatment.
Control.....	90% or more swim downward.	About 85% at bottom.	Number at bottom decreasing.	Scattered all over.	About 70% at upper part.	About 80% at top.
0.01 m. or 0.0133 m. $CaCl_2$.....	Abnormally active and 95% or more swim downward.	About 95% at bottom.	About 80% at bottom.	About 60% at lower part.	60% or more at upper part.	About 85% at top.
0.01 m. or 0.0133 m. Na_2CO_3 or $NaHCO_3$.....	About 95 % swim downward.	85% or more at bottom.	Scattered all over.	About 70% near top.	80% or more at top.	About same.
.05% HCl[1].....	90% or more swim downward.	70% or more at bottom.	Scattered all over.	About 60% at upper part.	About 70% near top.	About same.

writer that this means that the so-called reversible effect of chemicals on the negative geotropism of *Paramecium* is not specific but general. Moreover, the animals, whenever transferred to another receptacle without changing the solution, swim downward, and reversed animals do not stay long at the bottom.

There is, therefore, reason to believe that this is the same phenomenon that we met in the temperature experiments. The writer concludes, therefore, that mechanical "shock" or agitation of transferring, or chemical "shock," is chiefly responsible for so-called reversible effect of chemicals on the negative geotropism of *Paramecium* but not chemicals themselves.

Besides the chemicals mentioned above, sodium chloride,

[1] This was adopted as approximately Sosnowski's strength.

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magnesium chloride, cane sugar, ethyl alcohol and chloroform in appropriate strength were tried but no better results were obtained.

IV. Summary and Conclusions.

1. The specific gravity of living *Paramecium caudatum* is between 1.0382 and 1.0393.

2. *Paramecium caudatum* contains protoplasmic materials of different gravities.

3. *Paramecium caudatum*, when strongly centrifuged, assumes a position with its anterior end directed away from the axis of the centrifuge. In so doing, it must be passively thrown by the centrifugal force, because the centrifugal force used was altogether too strong to be resisted by the animal. For this reason, its anterior end must be heavier than its posterior end. The negatively geotropic orientation, therefore, is an active process and the mechanical theory is not correct.

4. The whole organism seems to be a "statocyst," since the recovery of negative geotropism after centrifugalizing is synchronous with the reëstablishment of normal relations of substances in the cell.

5. So-called reversibility of the negative geotropism of *Paramecium caudatum* by temperature and by chemicals is extremely doubtful. Mechanical "shock" or agitation is chiefly responsible for the reversal one sees in such cases.

In conclusion the writer wishes to acknowledge his indebtedness to Professor E. P. Lyon, under whose direction this work was completed, and who gave valuable suggestions and criticism of the work and manuscript. His thanks are due also to Dr. J. F. McClendon, who gave his generous help and suggestions during the experiments.

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NOTE ON THE GEOTROPISM OF PARAMECIUM.

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Harper's[1] criticism of my experiment demonstrating, as it seemed to me, that the anterior end of paramecium is the heavier and consequently that the negative geotropism of this animal is an active process was founded upon the assumption that when one begins to centrifuge the animals their geotropism is reversed and becomes positive. Thus the animals start, according to Harper, to move in the direction of the accelerating force and consequently away from the axis of the centrifuge. Harper's experiment by no means proves his contention and it always seemed to me, as Kanda brings out in the foregoing paper, that the quickness with which a high centrifugal force is developed in the centrifuge would prevent the animals from orienting themselves before they were thrown into the capillary tube where turning is impossible. However, this suggestion might not seem proof to some people; and I have, therefore, performed the following experiment:

A culture of paramecium was cooled almost to 0° C. At this temperature they move very sluggishly and show no distinct orientation to gravity. They gather at the bottom of a test tube due, I am sure to the fact that their specific gravity is greater than water and that they make no movement sufficient to keep themselves suspended. The centrifuge was prepared with capillaries such as Kanda and I have used but each one immersed in a larger tube containing ice. A drop of the cold paramecium culture was quickly transferred to the cold capillary tube and centrifuged. The animals were always precipitated with their anterior ends away from the axis. I am quite certain that the animals at this temperature make no reaction to gravity or agitation and that the proof may be considered definite that the head end of the animal is the heavier and that the ordinary geotropic orientation is an active process.

[1] For the literature see the preceding article by Kanda.

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THE MICROVIVISECTION METHOD.

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The dissection of living cells has hitherto proved inadequate for a study of the physical properties of protoplasm and of its structural components. This is owing largely to a lack of means for the manipulation of dissecting instruments under a sufficiently high magnification of the microscope.

Before and since Barber first devised his mechanical pipette-holder for the isolation of bacteria, various ingenious methods for the dissection of microscopic objects have been described. They all, however, fall short of Barber's method, because, with them, only comparatively low powers can be used, since the dissecting needles must operate *between* the objective and the tissue to be dissected. On the other hand, by using needles instead of micro-pipettes, Barber's instrument has been converted into an excellent microdissection apparatus. The needles operate in a shallow hanging drop containing the cells to be dissected which are pressed against the *undersurface of a coverslip* in a moist chamber. There being no obstacle above the coverslip, oil immersion lenses may be used for observation.

In an article appearing as long ago as 1859, Dr. H. D. Schmidt, of Philadelphia, describes in detail an excellent "microscopic dissector" consisting of a base to be fastened on the stage of a microscope with a number of clamps to hold instruments, each clamp possessing three movements controlled by screws. A lever fastened in one of the clamps holds the tissue in place. Fine scissors, knives or steel needles are fastened in the other clamps. By turning the various screws, the instruments can be brought into place and be operated with remarkable accuracy. Dr. Schmidt worked with the tissue, the instruments and the lower lens of the objective immersed in water or diluted alcohol. The full results of his investigations with this instrument were not published until 1869, owing to interruptions due to the Civil

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War, during which his drawings and manuscript were burned in a conflagration in Washington. In his later paper he has two illustrations showing a liver cell being torn in two by needles.

In 1887 Chabry described an apparatus in which a glass needle was held in a sheath in such a way as to allow the needle to be pushed to any prescribed distance into the bore of a glass capillary tube. The bore of the capillary was made just large enough to admit a single ovum and hold it in place. By proper adjustments, the needle could be pushed to any depth in the ovum. The apparatus was placed on the stage of a microscope and Chabry was able to injure locally a *Strongylocentrotus* ovum while under observation. Chabry's instrument has been used for experimental embryological work and is described in detail in Ehrlich's "Enzyklopädie für microskopischen Technik," 1910.

In 1907 J. F. McClendon first described his "mechanical finger," which consisted of an ordinary Spencer mechanical stage with an additional screw to allow of movements in three different directions and a clamp to hold a needle or pipette. With this apparatus, McClendon was able to suck the nucleus out of a *Chætopterus* egg. In 1909 he described an improved form with which he dissected certain Protozoa.

In 1912 an apparatus was described by Tschachotin. It consists of a clamp attached to the side of the objective of a microscope. In the clamp is fastened a needle which curves so as to project under the objective and is so adjusted that the tip lies in the focus of the objective. The needle point is lowered into the object to be dissected simply by bringing the object into view under the microscope and lateral tears are made on moving the object with the slide by means of the mechanical stage.

In 1904 Barber first described his method of using a hanging-drop in a moist chamber for isolating microörganisms. A fuller account appeared in 1907 in which he first describes a mechanism for holding micropipettes and where he mentions Montrose T. Burrows as having assisted him much in its design. Elaborations of his instrument appeared in his papers of 1908 and 1911. In 1911 he suggested the possibility of the use of his instrument for cell dissection and for investigation on fertilization and heredity problems. His paper of 1914 is a detailed and precise descrip-

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tion of his technique and is of the utmost value for the prospective worker in micro-dissection. As he developed his method principally for the use of pipettes and also because his latest paper appeared in a publication not readily obtainable, I have endeavored to give here a description of the method with modifications and new developments in the application of the method to microvivisection.[1]

A simple form of Barber's apparatus is shown in Fig. 1. The moist chamber, which is open at one end and with sides from 8 to

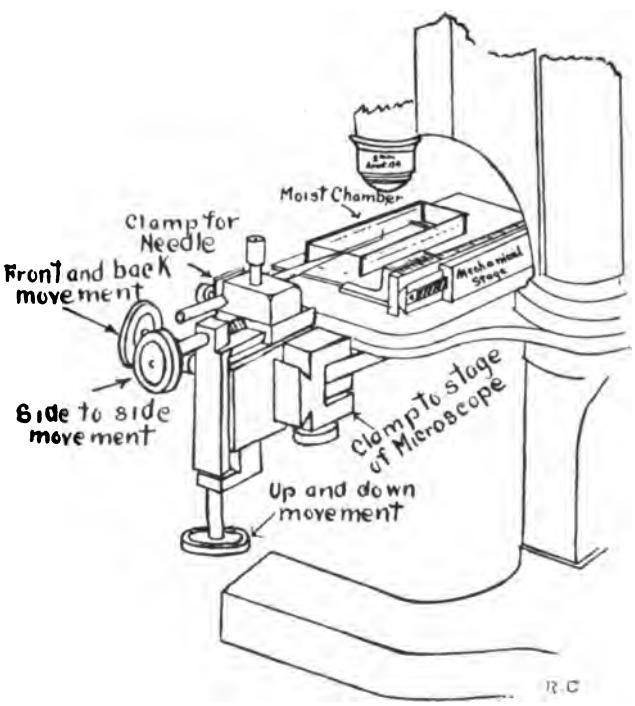

FIG. 1. Barber's single three-movement pipette holder, glass needle and moist chamber arranged to illustrate method of dissecting cells in a hanging drop under the highest magnification of the microscope. (Substage of miscroscope omitted in drawing.)

[1] In 1912, G. L. Kite first applied Barber's apparatus to microdissection, which quickly resulted in the publication of several papers. Kite was to have prepared an article on the method when his health unfortunately broke down. Publications on microdissection have appeared as follows: 1912, Kite (on the fertilization membrane) in *Science*, N. S., XXXVI.; Kite and Chambers (on chromosomes and nucleus) in *Science*, N. S., XXXVI. 1913, Kite (on permeability of cells) in BIOL.

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12 mm. high, is placed on the microscope so that it may be moved about with the mechanical stage. The chamber is roofed over with a carefully cleaned coverslip, on the under surface of which, the specimen is mounted in a shallow hanging drop of a physiologically indifferent fluid. The dissecting needle is made by drawing out one end of a piece of glass tubing and bending it at right angles two or three millimeters from the pointed tip. The needle-holder, a mechanism allowing of three movements, is clamped to one side of the microscope stage and the needle is adjusted so that it may project into the moist chamber with its tip pointing up into the hanging drop. By proper adjustment the cell to be dissected and the point of the needle are brought into the same focal field. The three movements of the needle, permitted by the needle-holder, and the two movements of the moist chamber, by means of the mechanical stage, give the experimenter ample opportunity to carry on dissection under the highest magnification of the microscope.

The Instrument.

The holder accommodated to carry only one pipette or needle is illustrated in Fig. 1. It is useful in many ways and is comparatively inexpensive. Its disadvantage lies in the fact that the resistance of the cell-substance to the needle may overcome the inertia of the cell, so that, instead of tearing through the cell, the needle simply drags it about. However, by careful manipulation and by the possible use of a viscous dissection-medium much can be done. Many marine ova and germ cells in general are very soft in consistency and, if the drop in which they are immersed be shallow enough, the surface tension of the drop flattens them against the coverslip and is sufficient to hold the cell. The single holder can also be successfully used for injecting material into or extracting material out of a cell. In addition to the movement of the needle, manipulation is greatly aided by the

Bull., XXV., and (on protoplasm) in *Amer. Jour. Physiol.*, XXXII., 1914, Chambers (on the nucleus) in *Science*, XL.; Kite (structural transformations of blood cells) in *Jour. Inf. Dis.*, XV. 1915, Chambers (on the germ cell) in *Science*, XLI.; and (on protoplasm) in *Lancet-Clinic*, March 27, Cincinnati; Kite (on cell permeability) in *Amer. Jour. Physiol.*, XXXVII. 1917, Chambers (on protoplasm) in *Amer. Jour. Physiol.*, XLIII.; and (on the cell aster) in *Jour. Exp. Zoöl.*, XXIII.

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use of the mechanical stage by means of which the moist chamber may be moved in two directions.

The double holder, formerly manufactured by the Fowler shops of the University of Texas, is twice as expensive as the single holder. It possesses, however, the advantage that two needles or pipettes may be used simultaneously. Each needle can be moved independently of the other and either needle may be replaced by a pipette. The manufacture of the holder has recently been duplicated by the mechanician of Wesleyan University under the direction of Dr. H. B. Goodrich with modifications of his and my suggestion. In setting up the apparatus, Dr. Goodrich introduced the useful innovation of having the shelf to which the instrument is clamped on a stand independent of the microscope. The two stands are then clamped together on a common base. The operator can thus shift the position of the instrument placing it at will in front of or on either side of the microscope.

Fig. 2 illustrates the double holder as I have it set up. The attachment of the instrument to the front of the microscope stage facilitates manipulation of the screws with both hands. In the illustration, a Spencer Lens Company lamp replaces the substage mirror. As the heat of the lamp, however, produces undue evaporation and subsequent condensation in the moist chamber, it is preferable to use the mirror. This necessitates raising the microscope-stand on a block of wood so that the mirror can be lowered below the microscope base so as to receive light unobstructed by the lower screws of the dissecting instrument.

The needle-carrier with its groove (Fig. 2, *a*), in which the arm of the needle lies, is twice as long as in Barber's original instrument. This extra length assists one in giving a straight even movement to the needle when it is being pushed into the moist chamber. After the needle has been thus adjusted by hand approximate to its proper position, the plate *b* is pushed over the arm of the needle and the screw *c* tightened to clamp the needle. Further adjustment of the needle is carried out by the screws *e*, *f*, *g* and *h* under the low and high powers of the microscope.

In preparing the shelf on which the instrument is to be clamped (Fig. 2, *j*) care must be taken that it be low enough to bring the top of the carrier flush with the upper surface of the microscope

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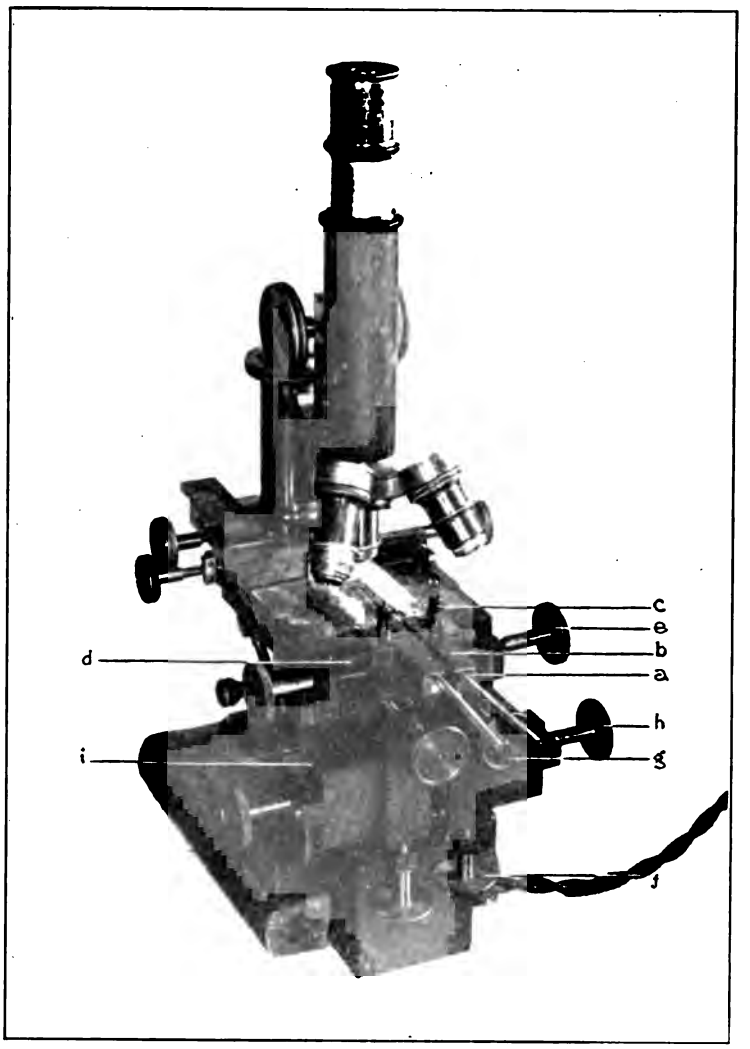

FIG. 2. Double pipette holder set up for microdissection. *a*, Carrier with groove to hold needle. *b*, brass plate which slips sideways over the groove in which needle lies. (The undersurface of the plate is covered with blotting paper to give it resiliency where it comes into contact with the glass needle.) *c*, set screw to clamp plate on carrier. *d*, screw which, on loosening, allows carrier to be set at any angle. *e–h*, screws to move the needle (*e*—side to side, *f*—up and down, *g*—in and out, *h*—side to side for both carriers together). *j*, shelf to which instrument is clamped.

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stage. This is in order to enable one to use a moist chamber of the minimum height (see below).

An important factor in the manufacture of the microdissection instrument is that the two horizontal movements run true and keep the needle point always in the same plane. Otherwise, at a critical moment in the dissection, a reversal of a horizontal screw may either suddenly lower the needle-point out of focus or jam it against the coverslip and break it. Unfortunately, the last model put out by the University of Kansas is deficient in this respect. The existence of lost motion in the screws can be taken care of in the manipulation but if the needle tip does not run true in a horizontal plane the peace of mind of the operator may be sorely tried!

The Moist Chamber.

The moist chamber (Fig. 3) is a glass slide to which are cemented strips of glass in such a way as to form a box open at the top and at one end. For cement Canada balsam may be used.

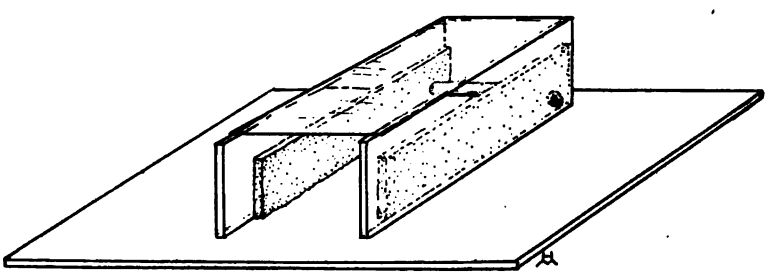

Fig. 3. Moist chamber for microdissection.

A shallow trough, to hold water, is made with a narrow glass strip set across the floor of the chamber 8 or 9 mm. from the closed end. This and other small strips set outside the chamber serve to reinforce the walls. Care must be taken that the upper edges of the chamber be even and level when placed on the stage of the microscope. A carefully grease-free cleaned coverslip with a hanging drop containing the objects to be dissected forms the roof of the chamber. The roof is sealed on with vaseline smeared along the upper edges of the walls of the chamber.

The height of the chamber is conditioned by the focal distance of the condensor used and by the minimum working room that

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one must have in which to manipulate the needles. For routine work it may be well to have a chamber of fairly large dimensions disregarding the condensor as it is surprising how much illumination a condensor will give beyond its focal distance. However, for critical work, it is imperative to have the height of the chamber such that the objects on the undersurface of the coverslip be close to the focus of the condensor. My condensor has a working focal distance of almost 9 mm. and the chambers I use are 45 mm. long, 22 mm. wide and 9 mm. high.

The moist chamber is lined on the sides with blotting paper leading from the trough to carry the water along the length of the chamber so as to furnish a large moist surface. The liquid in the trough and the blotting paper should be isosmotic with the liquid used for the hanging drop so that evaporation be equalized and the hanging drop not become diluted.

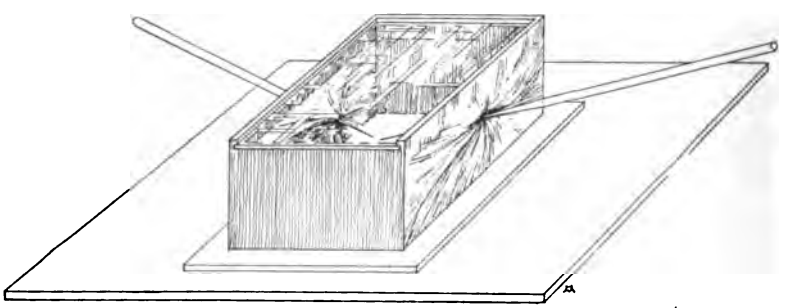

FIG. 4. Moist chamber for preliminary teasing of tissue.

For the dissection of the cellular elements of somatic tissues, it is necessary to do some preliminary teasing under an ordinary dissecting microscope. This should be done in a moist chamber of some kind. The tissue is teased on a coverslip which is then inverted and placed on the microdissection moist chamber.

A handy moist chamber (Fig. 4) can be made out of a shallow box with dimensions approximately 7 × 5 × 3 cm., with a top and bottom of glass and the two longer sides of thin rubber sheeting. The coverslip with the tissue to be teased is placed within the box and the teasing is carried out with needles projecting through the rubber sheeting. For the tissue of warm-blooded animals both the preliminary teasing and the microdissection should, of course, be carried on in warm boxes.

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Tissue cultures are grown on square coverslips. The slips are then placed on the moist chamber for microdissection, the parts of the chamber not roofed over being previously covered with slips of glass.

The Needles.

These are made from glass tubing with an outside diameter of about 4–6 mm. and a lumen of 3–5 mm. The technic is readily mastered with soft glass tubing but needles made of hard glass are more durable.

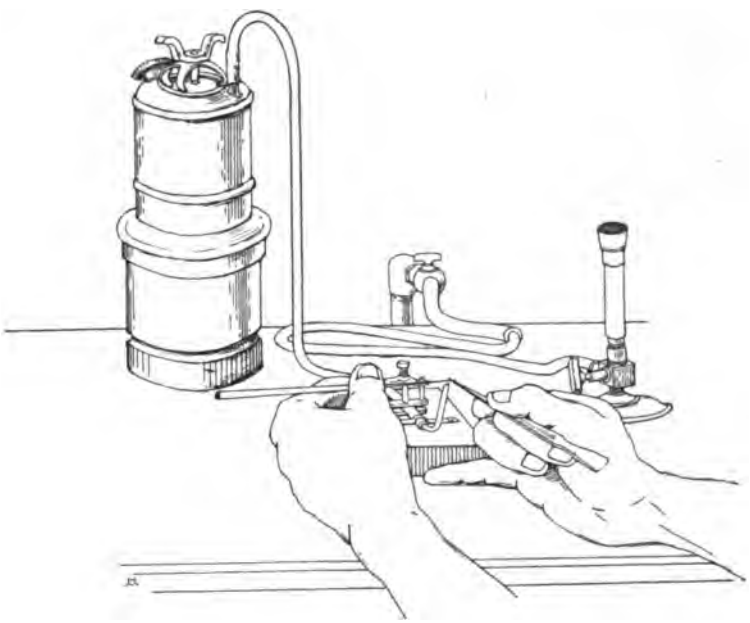

Fig. 5. Method of making the capillary pipettes and microdissection needles.

The needle points are made over a minute flame. A serviceable micro-burner with an acetylene generator is shown in Fig. 5. The microburner is made out of a piece of hard glass tubing bent almost at right angles and with one end closed except for the smallest possible aperture that will retain a flame. This is done by heating the end and pinching it with a pair of forceps. When set up the tip of the burner should be at a height of about 5–6 cm. above the surface of the table. The size of the flame is regulated by the screw pinch-cock set on the rubber tube connecting the burner with the gas generator.

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As a source for the flame acetylene is to be preferred to ordinary illuminating gas because with the former a narrower flame can be obtained without undue clogging. The acetylene generator in the figure is part of an old bicycle lamp. A more convenient way is to have the acetylene in a small compression tank which can be recharged at a very small cost. If, however, ordinary gas is to be used, one may much improve the gas by passing it through alcohol or benzene.

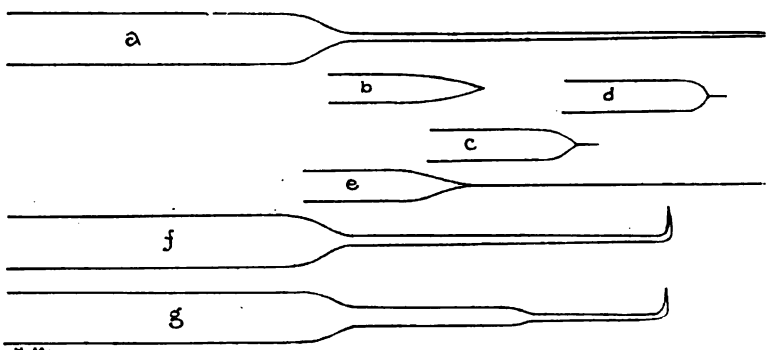

FIG. 6. Stages in making needles. *a*, glass tube with one end drawn out into a capillary. *b*, good tapering point. *c* and *d*, serviceable points. *e*, tip drawn out into a hair. *f*, completed microdissection needle. *g*, needle made on a relatively stout shank.

The method of procedure in making the glass needle is as follows:

1. In an ordinary Bunsen burner (or a Mecca burner if hard glass be used) draw out one end of a piece of glass tubing into a straight capillary about 0.3–0.5 mm. in outside diameter (Fig. 6, *a*). Prepare a supply of such pieces with the capillary ends at least 6 cm. long.

2. Lower the flame of the microburner to the smallest flame that will remain lighted. The microburner should be protected from draughts of air and in semi-darkness so that the flame may show up to best advantage.

3. Hold the shank of the glass tube in the left hand and, with a pair of fine forceps held in the right, grasp the capillary at a point about 5 cm. from the shank. Bring the portion of the capillary next the forceps over the flame and at right angles to it at a point just over the flame (Fig. 5). Pull gently with the

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forceps and, when the glass begins to soften, lift it slowly from the flame and pull with the forceps slightly more than at first, but not too strongly. The hands should remain on the table during the process and the pulling and lifting done by turning them slightly outward. The capillary will separate with a slight tug—a feeling much like that experienced when a taut thread, held in the fingers, is parted in a small flame. If the point is properly made, it will appear as shown in Fig. 6, *b*, *c* and *d*. This capillary has sufficient rigidity and it comes to a very fine point. The tip is closed, but the lumen extends almost to the end. It is evident that everything depends upon the amount of heat used and the timing of the pull, and that these must vary slightly with the height of the flame and the diameter of the capillary. With a little experience, one can usually tell when a proper point is made by the peculiar feeling described above. If too little heat is used and the pull made too suddenly, the capillary may part with a snap and the tip be found broken off short often forming a serviceable pipette. If too much heat is used, the capillary is apt to be drawn into a long hair (Fig. 6, *e*).

4. After a suitable point is made, the end of the capillary is turned up at right angles (Fig. 6, *f*). This is done by holding the point of the capillary just back of the point above the small flame and pushing up the end with the tip of the forceps or with a needle. Not more than 5 mm. should be turned if it is to be used in a chamber not over 8 mm. in height. If a good point is made, *e. g.*, Fig. 6, *b*, it is not necessary to turn the end at exactly right angles. A pipette is made by jamming the tip of a needle against the cover glass in the moist chamber to break off the point.

The forceps used in the making of the needles should be of a size that can be firmly grasped in the hand. The fine tips should be accurately set and almost parallel one another for a few millimeters. In order to give the tips a good, grasping, resilient surface I dip them, before using, in a small vial of Sandarac varnish which may be obtained from a dentists' supply dealer. As the varnish burns if brought too close to the flame, it is well to use a second forceps or a needle for bending the glass capillary.

Fine points are not readily made from capillaries of a diameter

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greater than .05 mm. Therefore, if the dissection requires a comparatively stiff shank for the dissecting needle, start with a glass tube possessing a thick capillary and draw out the tip into a thinner capillary. This thinner capillary may then be used for making the needle point (Fig. 6, *g*).

The facility in making the needle-points over the microburner increases, of course, with practice. In my case, I make at times twenty or thirty within an hour, at other times none, or only one or two at the most. A supply may be kept on hand on a stand made of two horizontal layers of wire netting, the meshes of which admit the base of the needles. It is well to mark off the stand into compartments to receive the various types of needles that are made.

Another good method for making the needle points is that of Chabry ('87). The tip of a capillary pipette is brought into contact with a heated mass of glass (or any incandescent body to which glass will adhere) and suddenly drawn away. For an incandescent body Chabry used the blade of a platinum knife of a surgical instrument for thermocautery. The glass capillary was held by the operator in a groove on a stand a few centimeters from the platinum blade, which was fastened so as to be immovable. An assistant controlled the heating of the platinum blade. As soon as it heated to a dull glow, the operator slid the capillary on its groove until the tip touched the glowing metal when it was instantly slid back. The sliding of the capillary in a stationary groove insures a tapering to a point in a straight line with the long axis of the capillary. In his paper Chabry discusses this method in some detail, pointing out ways of avoiding difficulties in the technique ('87, pp. 175–178). Chabry also suggested the use of insect mouth parts, annelidan bristles and the spicules of sponges for the tips of microdissection needles. Barber suggested fine-pointed needle crystals, or the sharp, stiff hairs taken from the body of a house fly. A pipette with a fairly large opening is made and the hair or crystal is drawn partially into it. The fine point projecting from the tip of the pipette is then used as a probe or dissecting needle. Such needles could be used only on very delicate soft objects.

Needle points may also be made by grinding one end of a fine

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wire. To produce chemical effects in a cell, McClendon ('09) used a copper wire ground to a point and further sharpened by erosion in acid. The chemical injury produced by metal needles limit their use for the dissection of living cells.

For experiments in cutting cells, one must take into account the fact that many cells will lose their more or less fluid contents if a gap be torn in their surface. If, however, the side of a fine needle can be brought to press the cell against the coverslip, a constriction will result which may cut the cell into two intact pieces. For cutting soft-bodied ova and protozoa this can be done with a form of needle depicted in Fig. 7. The needle tip

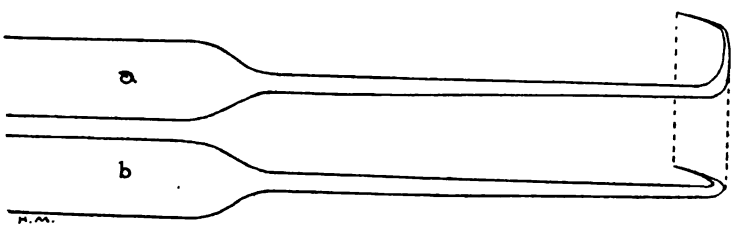

FIG. 7. Needle with tip curved back to almost an horizontal plane. *a*, lateral view. *b*, view from above.

is bent back rather than forward to lessen the chances of breaking the tip when adjusting the needle in the moist chamber and also when the needle needs to be cleaned (see below). The tip should not point directly back but slant off to one side (see Fig. 7, *b*), so that the shank of the capillary will not be in the way of the light coming up from below. The angle at which the needle is bent must be ascertained by successive trials, for, if it be bent too near to a horizontal plane, the tapering tip will bend away from the cell instead of cutting into it. If the angle be too far from the horizontal plane, the cell will slip along the needle and escape. When the proper angle is obtained, one can cut *Arbacia* and *Asterias* ova into pieces of almost any required size with considerable ease. A clean cut through an unfertilized sea-urchin egg or a fertilized egg, before the fertilization membrane has become too tough, can be best made by bringing the egg between the horizontal arm of the needle and the lower surface of the hanging drop (Fig. 8). On lowering the needle out of the drop,

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the arm of the needle passes through the egg cutting it cleanly in two.

A good needle, when once made and proven to be resilient and strong, may be used repeatedly. Mucinous material which soon

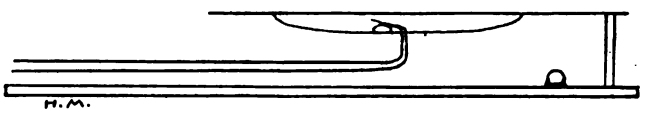

FIG. 8. Side view of moist chamber to show method of cutting an egg cell in two with needle shown in Fig. 7.

covers its tip so as to render it useless can be got rid of by immersing the needle successively in a strong acid and a strong alkali.

CELL INJECTION.

Barber, in his two papers ('11 and '14) gives a full description of his admirable mercury method for injection. With it one can inject microscopically minute doses of a solution into any part of a cell. The advantage of the pressure produced by the mercury over that by air lies in the slow driving force of expanding mercury which can be almost instantly arrested when the dose injected is considered sufficient. On the other hand, if air pressure be used, the air in the injection tube will compress enormously as the resistance of the bore at the tip of the pipette is too great to overcome. If the injection does occur it may go with a rush usually followed by a volume of air which destroys the cell being experimented upon. Notwithstanding this, it is surprising what delicate work may be done simply with air pressure. An example of this is the extraction of the nucleus from a mature *Arbacia* egg and its subsequent injection into another egg. For the injection of substances, however, where the amount must be strictly controlled, the mercury method is essential.

The extraction of material out of a cell is comparatively easy to carry out, as the capillary attraction in the lumen of a micropipette (the inside diameter of which may be anywhere from 2 micra up) is quite sufficient for the purpose. For injection purposes the difficulty lies in securing the force to overcome this capillary attraction.

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Source of Light and Lenses.

A most serviceable light for microscopic work is the 100-watt horseshoe filament, Edison, Mazda, nitrogen lamp with the blue daylight glass. This lamp, when set in a frosted milk-white glass globe, gives a white light very restful to the eye.

In selecting lenses care should be taken to procure those possessing the maximum focal length together with the greatest numerical aperture. For this reason the Zeiss apochromatic 4 mm. dry lens with a N. A. 0.95 and the 3 mm. oil immersion lens with N. A. 1.40 are excellent for microdissection or for the study of living tissue in general. It is to be remembered that the equivalent focus of a lens does not correspond with the working focal distance.

A good substage condensor with a working focal distance of 8–9 mm. can be made by removing the top lens of the Leitz triple-lens, centering condensor. The beam of light issuing from it completely fills the back lens of a 4 mm. objective with N. A. 0.65 and more than half of a 1/12 oil immersion objective with a N. A. 1.30. This gives enough definition for such detailed cytological work as the study of the chromomeres in grasshopper germ-cells and of the types of protoplasmic granules in marine ova. One may obtain very good condensors with a long working focal distance from those used in projection apparatus where a cooling trough is inserted between the condensor and the microscope stage.

Setting up the Instrument.

The instrument is first clamped to the microscope and the moist chamber, with the edges of its walls smeared with vaseline, placed in the mechanical stage. The needle is then adjusted as follows: Place the arm of the needle in the groove of the carrier with the dissecting tip extending into the moist chamber. Push the plate of the carrier (*b*, in Fig. 2, page 126) over the arm of the needle and tighten the set screw *c* just enough to allow one to push the needle with a straight and even movement. The needle is now adjusted by hand so as to bring the tip within the field of a low power objective. The set screw is then tightened to clamp the needle in place. Further centering of the needle is made by

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the screws *e*, *f*, *g* and *h*, under the low and high powers of the microscope. Finding the tip of the needle under high powers is facilitated by using an ocular containing cross hairs.

The coverslip with its drop containing the tissue to be dissected is now inverted over the moist chamber. The cells to be dissected are brought into the field of the microscope. The tip of the needle is then raised by the screw *c* until brought into view first under low and then under high power. The apparatus is now ready for dissection.

In conclusion I wish to suggest to the prospective worker in this field that he use the utmost conservatism in his interpretations of observed effects produced in microdissection. One must constantly keep in mind the extraordinary instability ot living cell structures.

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Vol. XXXIV. *March, 1916.* *No. 3*

BIOLOGICAL BULLETIN

THE LOCOMOTIONS OF SURFACE-FEEDING CATERPILLARS ARE NOT TROPISMS.

C. H. TURNER,

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PRELIMINARY OBSERVATIONS.

While collecting cherries from a tree a large number of apple-tent caterpillars were thrown upon the ground. Later several of these were noticed climbing the trunk of the tree. They continued onward and upward; some to the top of the tree and others to certain branches. Near the tree was a rustic chair, the top of the curved back of which was separated from the trunk by about three inches. Several caterpillars climbed the back of this chair. When they reached the top, they would elevate the front portion of the body, wave it back and forth and around for a few minutes and then proceed down the opposite side of the curved arm. Sometimes the insect would turn about and retrace its steps. These observations suggested two questions. Are these caterpillars guided by a negative geotropism? If so, why did those that had reached the top of the chair descend?

TECHNIQUE.

These experiments were performed upon a wooden vertical maze (Figs. 1, 2) and a copper horizontal one (Fig. 3); both of the open type. The former was used in the field and the latter in an out-door insectary.

The vertical maze (Figs. 1, 2) consists of a cylindrical post about four feet tall and an inch and a half in diameter, to which are attached five adjustable rectangular arms. Some of these arms are straight and unjointed; others are formed of three

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straight sections so articulated as to form an arm with three adjustable sections. The straight arms and the sections of the longer arms are all of the same length. The adjustments of the arms make it possible to set each and every one at any desired angle to the pull of gravitation. The lower pointed end of the post was erected in loose soil in such a manner that, by rotating the post on its axis, it was possible to vary the position of any arm with reference to either the direction of the rays of the light or the path of the wind.

The copper horizontal maze (Fig. 3), which was supported on slender glass pillars, had one of its central alleys connected with the supporting table by means of a cardboard incline two inches wide and thirty inches long. It was so located that the strongest light came from one side.

The point of view expressed in this communication was obtained by a critical study of 1,000 caterpillars of the apple-tent moth, *Malocosoma americana* Fab., 300 of the cabbage butterfly, *Pieris rapæ* Linn., fifty of the white tussock moth, *Memerocampa leucostigma* A. & S., and a few individuals of the young of each of a large number of species of butterflies and moths collected from the surfaces of a large variety of food plants. Throughout a summer practically every kind of surface-feeding caterpillar that a diligent search of the vicinity of St. Louis yielded was put to the test. Among those tested were the flat slug-like Lyncenidæ, the elongated Papilionidæ and Pyralidæ. and the fusiform Hesperiidæ; the naked Noctuidæ, the hairy Arctiidæ, and Liparidæ, and the bristly and spiny Ceratocampidæ and Nymphalidæ; the Notodontidæ with their erect anal somites and the Sphingidæ with their oblique stripes and caudal horns; the grotesque Saturniidæ and the looping Geometridæ.

Experiments with the Vertical Maze.

To facilitate description, the parts of the vertical maze were named as follows: The upright was called the post; each straight arm and each section of a jointed arm was given a distinctive letter. Thus (Fig. 1) the first arm above the ground was called *abc*, the second, *def*, the third *j*, the fourth *g* and the fifth *h*.

In the first few hundred experiments with the tent-caterpillars

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the arms of the maze were arranged in a variety of ways. In the latter experiments with them and in the experiments with all others the maze was arranged as in Fig. 2; but, the bands *G* and *H* were used only on special occasions. In some of the experiments the plain maze was used; in others one or two odorous bands of filter paper were attached to certain portions of the maze. In experimenting one or more caterpillars were placed on the ground at the foot of the post, or else upon the top of the post, or on some intermediate portion of the maze.

When placed on the ground at the foot of the plain maze, the tent-caterpillar almost invariably letisimulated. In the first few experiments with the tent-caterpillars, on recovering from the feint, the larva moved toward and up the post. If its initial movements were away from the post, after a few steps it turned about and moved towards the maze. This striking behavior recalled some experiments by which H. B. Weiss[1] discovered that certain water insects when turned loose several yards from their home, would immediately go directly to the pond. It looked as though these caterpillars were being mechanically led to the post. To test the matter, a series of experiments, extending over several days, were performed. In these experiments precautions were taken to see that the heads of the caterpillars pointed in a variety of directions. In some cases the majority of the larvæ moved towards the post, in others the majority moved away from the post, in yet others about as many went away from the post as toward it. Evidently the behavior noticed in the first experiments was merely a coincidence, the uniformity of which may have been due to some factor which escaped observation.

Once upon the maze, each caterpillar displayed marked individuality. It might continue all the way to the top of the post without mounting any of the arms; it might, on its way up, explore a few or all of the arms; or it might turn about before reaching the top and return to the ground. At intervals it was sure to pause and reach upward and outward with the front end of its body and move it, in a wabbly manner, from side to side. [For

[1] Notes on the Positive Hydrotropism of *Gerris marginatus* and *Dineutes assimilis*. Canad. Entom., Vol. XLVI., pp. 269–271.

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brevity throughout this discussion this form of behavior is called exploring movements.] Should it reach the top, it might encircle it, pausing now and then to make exploring movements; or it might rest there for a long stretch of time. However, it would be more apt to descend the post, pausing now and then to make exploring movements. It might continue along the post

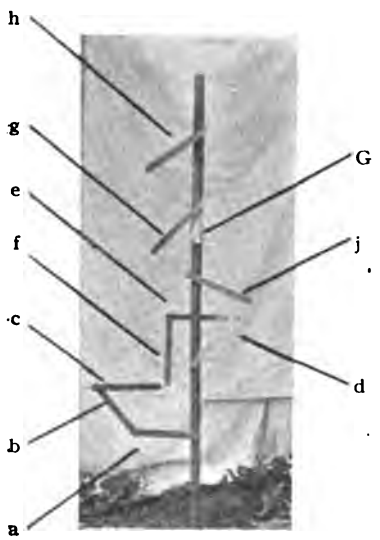

FIG. 1.

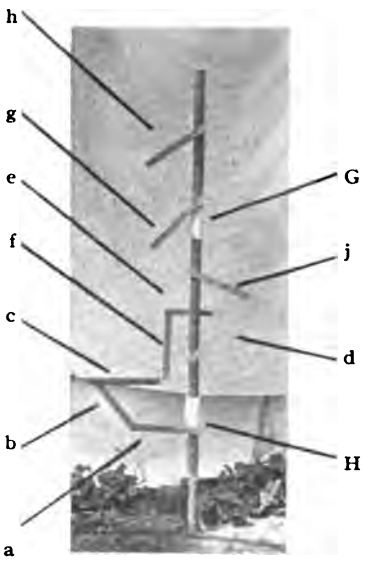

FIG. 2.

to the ground; but it would be more apt to make side trips to one or more of the arms. Often the same arm would be traversed several times. Finally it would rest for an indefinite length of time upon some part of the maze, or else reach the ground. Some caterpillars would leave the post permanently as soon as they reached the ground the first time. Others would remount the maze and explore it one or more times before leaving. The transcription of the field notes of two cases will illustrate the above description.

A tent-caterpillar about one inch long is placed on a maze arranged as in fig. 1; but without the band *G*. Ascending in a sinuous path, it passes around the bases of *a* and *d*, over the base of *j* and around the foot of *g* and *h* to the top of the post. The

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unhesitating manner in which it continues up and up is enough to rejoice any stanch believer in negative geotropism as the guiding power. Frequently pausing to make exploring movements,[1] it encircles the top a couple of times and then descends to *h*. It moves along *h* to its tip, makes exploring movements, returns to the post, descends to *e*, pausing several times to make exploring movements. It passes along *e* to *f*, makes exploring movements, descends *f* to its tip and makes exploring movements towards *c*. It reascends *f* to *e*, makes exploring movements, and returns to the tip of *f*. It makes exploring movements and then moves across the gap to *c*. It moves along *c* to *b*, along *b* to *a* and down *a* to the post. After making exploring movements, it encircles the post halfway, descends to the ground, moves off about three feet and rests indefinitely.

In this experiment, the subject of which is a tent-caterpillar, the arms *j*, *g*, *h* extend obliquely upward, on the left side of the post, at an angle of thirty degrees; *d* extends horizontally to the right; *e*, almost vertically upward, and *f*, almost horizontally back to the post; *a* extends obliquely upwards toward the left, at an angle of thirty degrees; *b*, horizontally towards the left; and *c*, obliquely upward, at an angle of about thirty degrees.

Following a path which is sometimes straight and sometimes spiral, the caterpillar continues upward until it reaches *g*, which it ascends to the tip. There it spends several minutes making exploring movements; then descends *g* to the post and crawls up it to the top. It goes about half way around the circumference, then crosses over to the opposite side and descends the post. Pausing occasionally to make exploring movements, it continues downward to *h*. It ascends *h* to its tip, crosses over to the opposite side and returns to the post. It continues downward an inch, rests a few minutes. Occasionally pausing to make exploring movements, it continues spirally downward to *g*, which it ascends for about four inches and upon which it rests quietly a few minutes. Returning to the post, it continues downward

[1] When a caterpillar reaches the end of branch or twig, when it encounters an angle or an obstacle, and sometimes for no visible reason, it pauses, elevates the front portion of its body, stretches it forward and upward (sometimes slightly downward) and waves it with a wabbly movement. For the sake of terseness in descriptions, this form of behavior has been called "exploring movements."

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to *j*, makes exploring movements, moves up and down *j* several times and then returns to the post. In a sinuous line it continues down the post to *d*, which it mounts. Returning to the post, it continues downward to *a*, which it mounts, makes a few exploring movements and, returning to the post, continues downward to the ground. It then reascends the post a few inches and rests a few minutes. It continues upward to *d*, moves out *d* to *e* and rests thereon indefinitely.

If one may speak of types where there is so much individuality, the above descriptions may be considered typical of the many hundred of records. There were, to be sure, specific variations. Some species were sluggish and others rapid, some were vigorous, others fatigued quickly, some were persistently seeking a solution of the difficulty, others rested at frequent intervals and for long lengths of time, or else made practically no attempt to solve the problem; some letisimulated for a long period when placed at the foot of the maze, others began to climb at once. All these, however, are but minor variations, the essentials are as stated above.

When a caterpillar is placed on top of the post, either immediately or after a slight letisimulation, it moves down the post; making the same variety of random movements as are made by those caterpillars that are placed at the foot of the maze.

When a caterpillar is placed on one of the arms of the maze, either with or without a preliminary letisimulation, it soon reaches the post. There it may either descend or ascend and make the same type of random movements as is made by caterpillars placed at the foot of the maze.

As a variant, one or more bands of filter paper, saturated with oil of cloves or oil of cedar, were attached to the maze at *G* and *H*, as indicated in Figs. 1 and 2. When one scented band was used the caterpillar was placed either at the foot of the maze or on its top. Whenever the insect reached the scented band, it made exploring movements and then retreated. Otherwise its behavior was the same as that of a caterpillar on a plain vertical maze.

Where two scented bands were used (Fig. 2), the caterpillar was always placed on the maze between the two bands. The only way for the captive to escape to the ground, without crossing a

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scented band, was to cross the gap between *f* and *c* and proceed along *cba* to the lower portion of the post. Roaming at random to and fro over the maze, pausing from time to time to make exploring movements, retreating whenever it encountered a scented band, sooner or later the caterpillar would usually cross the gap between *f* and *c* and escape along *cba* to the lower post and thence to the ground. Occasionally a caterpillar would drop from the maze to the ground; in a few cases, after a few movements, it would rest indefinitely in one place; in less than one per cent. of the cases, the insect would escape across the scented band. In these cases the band had not lost its odor; for immediately thereafter another caterpillar, on reaching the same band, would retreat. Even the same caterpillar, on being replaced between the bands, would retreat from the band across which it had recently escaped.

A transcription of the record of one experiment will illustrate the behavior under these conditions. The specimen is a tent-caterpillar and the maze is arranged as in Fig. 2. The bands *G* and *H* are moistened with oil of cedar. It is between three and four in the afternoon and the maze is situated in the shade of trees; but, here and there, blotches of sunlight touch it. The caterpillar, which is placed near *j*, ascends the post until it reaches the band *G*. There it makes exploring movements and retreats down the post. On reaching *j*, it partly explores it, then returns to the post, and continues downward to *e*. It moves along *e* to *f*, makes a few exploring movements, descends *f* and crosses the gap to *c*. Returning to *f* it ascends *f* to *e* and then retreats to the lower end of *f*. After making exploring movements, it crosses the gap to *c*, moves along *cba* to the lower portion of the post and thence to the ground. *The caterpillar is returned to its former position between the two bands.* It descends to the band *H*, makes exploring movements and retreats up the post. It mounts *j*, and, after making exploring movements, returns to the post and descends to *H*. After making exploring movements, it encircles the post and ascends to *e*. It mounts *e*, then returns to the post and continues upward to the band *G*. After making exploring movements, it descends to *j*, makes exploring movements, returns to the post and continues down-

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ward to *e*. It moves across to *f* and back to the post. After ascending the post a short distance, it returns to *e*, passes along *e* to *f*, climbs down *f* to its end and makes exploring movements. It then ascends *f* to *e*, passes along *e* to the post and ascends it to the band *G*. After making exploring movements, it descends to *e*, passes along *e* to *f*, where it rests several minutes (in the bright sunlight). It then descends *f* to its end and reascends it to *e*. Then descending *f*, it passes across to *c*, passes along *c* to *b* and then retreats as far as *e*. It makes three trips back and forth between *e* and *f* and then passes from *e* to the post, and up it to band *G*. Avoiding the band, it encircles the post about three fourths its circumference and then falls to the ground.

With the tent-caterpillars these experiments were conducted either in the shade, or in shady places that were spotted with sunlight that had filtered through the trees. With many of the others the experiments were conducted in the bright sunlight. An accerelation of movements and an apparent tendency to climb or descend the shady side of the post were the only differences noted in the behavior of the caterpillars under these conditions.

Experiments with a Horizontal Maze.

The maze used has (Fig. 3) been fully described in Vol. XXV, pp. 348–349, of the Biological Bulletin. A cardboard incline thirty inches long and two wide connected one of the interior alleys of the maze with the supporting table. Eight inches of this incline extended above the maze (Fig. 3). This gave each caterpillar that arrived at the incline a choice of two pathways; one passing obliquely upwards, the other obliquely downwards. The maze was placed in an out-door insectary, all the light of which came through the netting covered north side. In the earliest experiments two caterpillars were used at a time, and they were so located that they would be forced to approach the incline from opposite directions. Later, when attention was focused upon the behavior of the caterpillar when coming in contact with the incline, twelve to fifteen insects were used at a time; and they were placed on various parts of the maze.

Although the insects displayed great individuality, the be-

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havior in all cases was similar. They moved at random about the maze; roaming into blind alleys and out again, often retracing their steps when in the right pathway, and frequently pausing to make exploring movements. The rays of light were crossed

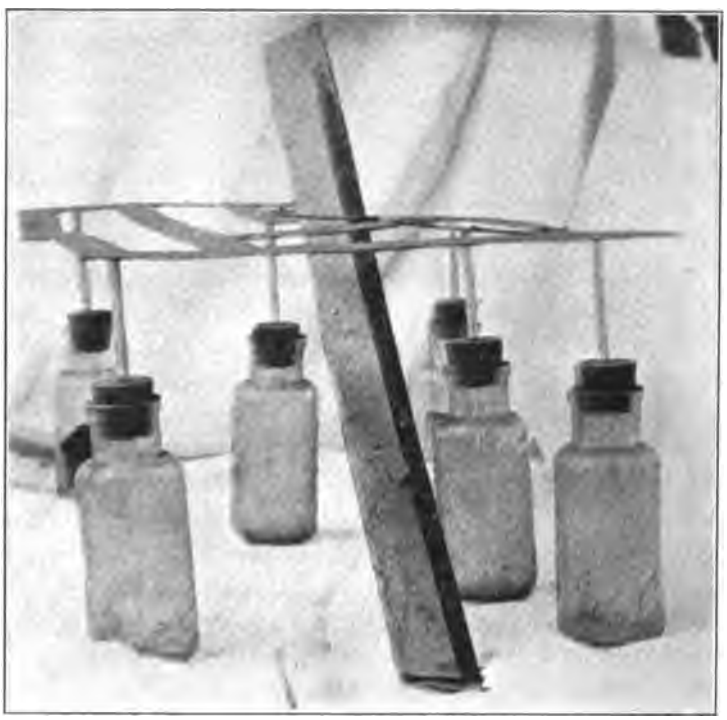

FIG. 3.

at every possible angle. When a gentle breeze was blowing, there was not the slightest indication of an anemotropism. There was one striking uniformity. Whenever a caterpillar came in contact with incline for the first time, it practically invariably ascended it.[1] This happened when the contact was made where the edges of the maze and incline came in contact, and also when the exploring movements of the insect brought it in contact with some portion of the incline that was higher than the maze. After once mounting the incline the caterpillar

[1] Only twice during the summer, did a caterpillar descend the incline upon its first contact with it.

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would often return to the maze, roam around and then remount the incline. With a few exceptions, the caterpillar finally reached the table and departed. In a few cases it reascended the incline after reaching the table, and in yet fewer cases the caterpillar finally rested indefinitely upon some portion of the maze.

In spite of the individuality, there were four things common to the behavior of all of the caterpillars placed on the horizontal maze. (1) The caterpillars usually traveled along the edges of the maze, instead of along the middle of the roadway. (2) At frequent intervals the creature would stop, cling to the maze with its prolegs, stretch the front part of the body forwards and dorsalwards and wave it about in a wabbly manner. (3) On reaching the incline for the first time, it practically invariably moved upwards. (4) In all cases the locomotions were random movements. Any interpretation of the behavior on the maze must be in harmony with these four factors. The first and the third are the only ones suggesting that the reactions of these caterpillars are tropisms. The almost constant clinging to the edges of the maze suggests some form of a positive thigmotropism; the invariable ascension of the incline upon its first encounter seems to predicate a negative geotropism.

If the clinging to the edges of the maze is indicative of a positive thigmotropism, why does not the caterpillar show a pronounced tendency to linger in the many angles of the vertical maze? Why does it not rest indefinitely in the angle between the upper portion of the cardboard incline and that alley of the horizontal maze upon which it rests? Would it not be more consistent to believe that the caterpillars move along the edge because they secure a better foothold there than upon the smooth surface of the copper maze?

The fact that the incline was invariably ascended when first encountered requires critical analysis. An examination of the notes made at the time of the experiments shows that whenever the caterpillar upon the maze came in contact with the incline its head was directed towards its upper edge. A thoughtful consideration of the physical conditions of the horizontal maze used and of the normal movements of a caterpillar on the maze shows that such a condition is inevitable. Should a caterpillar

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be beneath the upper portion of the incline and elevate the anterior portion of its body to make the exploring movements which invariably accompany near contact with a change in its environment, it would be almost certain to touch the incline, and its head would inevitably be pointed towards the upper edge of the incline. Should the caterpillar approach the incline where one of its edges touches the maze and make the usual exploring movements what would happen? If it moved its uplifted body inwards it would come in contact with the incline and its head would be directed towards the upper edge of the incline. Should it move its body outwards, since the incline descends abruptly, the caterpillar would come in contact with nothing and would then wave its body inward. Should the sweep be sufficiently long, the caterpillar would surely come in contact with the incline and its head would be pointed towards its upper edge. This suggests that it is not necessary to predicate a negative geotropism to account for the upward movement of the caterpillar on its first contact with the maze. It simply moves off in the direction that its head happens to be pointing when it encounters the incline.

To test the soundness of this conclusion, the following simple experiment was devised. A caterpillar was placed in a long narrow test-tube; which was sufficiently wide to permit freedom of longitudinal locomotion, but which was so narrow as to make it inconvenienient for the insect to turn around or make exploring movements. This device made it possible to induce the caterpillar to come in contact with the incline with its head pointing in any desired direction. It was only necessary to wait until the caterpillar was moving freely along; and then, after first pointing the mouth of the test-tube in the desired direction, to bring it in contact with the upper surface of the incline. In more than 99 per cent. of the cases, the insect moved off in the direction its head was pointing.[1] By means of this device hundreds of caterpillars were induced to move downward, upon their first contact with the incline.

This also explains why a caterpillar approaching the vertical

[1] In the few exceptions, I had waited too long and the caterpillar had curved its head around the rim of the test-tube before I had brought it into contact with the incline.

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maze from the ground always moves upwards, while one placed on the tip of the maze always moves downward. It is not necessary to postulate a negative geotropism to explain one and a positive geotropism to explain the other, and puzzle our brains to account for the reversal of the tropic response when the insect is bodily transported from one place to the other. The behavior is not a tropism. When the caterpillar nears the foot of the maze, the exploring movements which it invariably makes when it nears a change in its environment would cause it to first encounter the post with its head pointed upwards. When placed on the top of the maze, if its movements bring it in contact with the side of the post, it will inevitably be with the head of the insect pointing downward. In one case the insect ascends, in the other it descends; in neither case is the predication of a tropism necessary to explain the behavior.

Conclusion.

Evidently there is nothing about the behavior of surface-feeding caterpillars which warrants the assumption that their locomotions are tropisms. The movements made in locomotion are identical with those made by animals that learn by the trial and error method. Instinctively the caterpillars are physiologically attuned to a certain environment. Outside of that environment there is physiological unrest. To escape the unpleasantness of its environment, the creature makes random movements similar to those made by creatures that learn by the trial and error method. There is no automatic adjusting of the body so as to have it symmetrically stimulated by an external excitant. Some internal, instinctive stimulus causes it to move ahead until some sensation factor induces it to change its course. If physiological satisfaction is not obtained, it continues its random movements until fatigue causes it to rest. Evidently the locomotions of surface-feeding caterpillars need to be classified with trial and error movements rather than with tropisms.

It must be distinctly understood that nothing stated above refers to recently hatched caterpillars, nor to larvæ immediately before entering the pupa state, nor to caterpillars (like the coddling moth larva) which feed inside of plants. Their behavior is left for a future discusions.

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FACTORS INVOLVED IN THE ATROPHY OF THE ORGANS OF THE LARVAL FROG.

WITHROW MORSE.[1]

From the Nelson Morris Memorial Institute for Medical Research of the Michael Reese Hospital, Chicago.

PRELIMINARY.

Throughout the two great branches of the organic realm, instances are presented where persons, organs or tissues or cells themselves undergo retrogressive changes.[2] In the plant kingdom, we have the familiar elimination of stems, leaves and other parts of the individual, upon the approach of untoward conditions. In the animal series, we are enabled to identify an analogous process in the protozoa where, in *Amœba* the ephemeral pseudopodia are constantly being absorbed into the protoplasmic mass proper; in the species of *Actinophrys*, where this process of absorption is delayed permanently or at infrequent intervals; in the genus *Trypanosoma*, where the undulating membrane becomes absorbed under certain conditions. Similar phenomena are observed in the sponges where Maas's studies have shown that hitherto highly differentiated cells become reduced to a more typical and fundamental cell-form, the amœbocyte. In the Cœlenterata we find the hydranths of *Tubularia* (1) and of other species either absorbed or eliminated *in toto*, while in *Renilla* Wilson has described a degeneration of the polyp. The "brown bodies" of the Bryozoa represent degenerated individuals. In brachiopods, during the stages of fixation there is present an extensive degeneration of parts resembling in a superficial way the various changes undergone by the larvæ of the ascidians. In *Sacculina* we have, *facile princeps*, this property of involution. Even amongst the vertebrates we find instances of important degenerations of a natural sort, as in the fish *Fierasfer* where Bykowski and Nusbaum discovered extensive degeneration processes when the fish became parasitic.

[1] In order to avoid confusion, the author's name will be used henceforth as in the title of this paper.

[2] Compare Child, C. M., "Senescence and Rejuvenescence." Chicago, 1915.

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Interesting as these instances are, relatively meagre investigation has been made as to the causes and factors involved in the physiology of the process. On the other hand, such studies as have been made have borne good fruit. It was in the studies of ctenophore larvæ that Metchnikoff conceived the idea of what later became the theory of phagocytosis. Later this investigator studied the cytology of the larval frog's tail during the stages of metamorphosis. Extensive morphological studies have been made upon the stages of metamorphosis in insects. The changes which the salmon undergoes in breeding season have likewise received considerable attention.

Passing from the natural atrophy found in such instances as these, to what is termed pathological atrophy, we encounter the processes of progressive muscle atrophy, acute yellow atrophy of the liver and other states which bear many features in common with certain of the so-called natural atrophy processes, and this is true to such an extent in regard to the atrophy of the muscles of the larval frog at metamorphosis and the conditions observed in progressive muscle atrophy or Zenker's degeneration that it is difficult to believe that we are dealing with fundamentally different physiological processes.

The purpose of the following communication is to attempt to show that there are certain factors involved in the atrophy of the tadpole's tail which are to be considered as causal or of primary importance as conditions; and the similarity in cytological pictures and in certain known conditions in this case and in that of pathological atrophy makes it fairly probable that we are dealing with the same physiological factors, so that we encounter here an instance where the comparative physiology of a lower form of organism sheds light upon the economy of the higher organisms of especial interest to the pathologist.

Historical.

Although Metchnikoff had witnessed the activity of phagocytic cells earlier, in the larvæ of errantiæ, his first report involving the conception of cells wandering in the body fluids of one organism and serving as scavengers was made in 1883 (2). Early in the history of the problem, this investigator recognized the practical

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value of such a study when he says "L'atrophie des muscles des batraciens présente un grand intéret parce qu'elle peut servir de type de phénomenes pathologiques."

Following Metchnikoff, several investigators concerned themselves with the problem. Thus Barfurth (3), Looss (4), Noetzel (5), Anglas (6), Guieweisse (7), Mercier (8) are among the more important names in this connection. Of these, Looss and Mercier are perhaps of the greater importance. Grund (9) has more recently studied the problem of muscle degeneration from the chemical standpoint. In the field of fish physiology, Miescher's (10) classic researches and those of Noel Paton (11) and his coworkers demand especial mention. Even mere mention of the investigations which have been made in insect physiology and morphology during metamorphosing periods is impracticable in the present paper, a review of which being available in the paper of Mercier (8).

The Problem Stated.

Two quite diverse theories concerning the factors involved in muscle atrophy exist, namely, *phagocytosis* and *autolysis*. The former theory can best be stated in the words of Metchnikoff: "Von den ersten Stadien seiner Atrophie an, kann man in ihm eine gross Anzahl amöboider Zellen finden, in deren Innerin ganze Stücke von Nervenfasern und Muskelprimitivbündeln enthalten sind" (1883, p. 561). Looss adheres to the latter view in the following words: "Wir es hier nicht mit einer Degeneration, einer Entartung der Gewebe und ihrer histologischen Bestandtheil zu thun haben, sondern mit einer reiner Auflösung, mit einer Resorption im strengen Sinne des Wortes" (p. 91).

In the larval frog, no one has attempted to verify the belief of Looss save upon histological grounds. Anglas, Noetzel, Guieweisse and others subscribe freely to Metchnikoff's theory of phagocytosis. The burden of the present paper is to show that Looss is correct in believing that fundamentally and primarily, a change is initiated interpretable as autolysis and that phagocytosis, which unquestionably is present at a later stage, is of secondary importance.

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Histological Evidence.

In the accompanying photomicrographs, evidence is presented that events have occurred in the tail of a larva before the advent of phagocytosis. Fig. 1 is that of a section through the larval

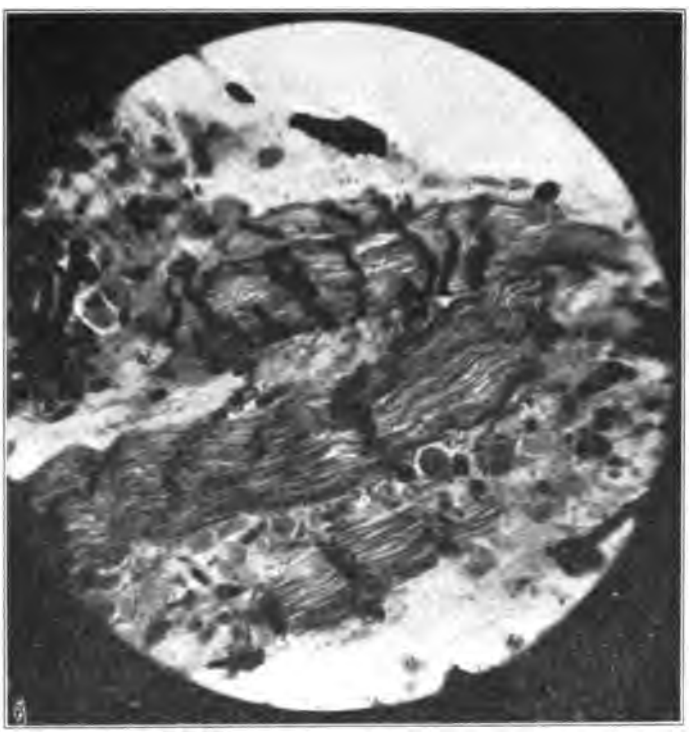

Fig. 1. Section through the tail of an involuting *Rana pipiens*. Zeiss 2 mm. obj. ocular 6. Osmium tetroxid fixation. Heidenhain's ferric alum-hematoxlyn.

frog's tail, lateral and ventral to the nerve and vertebral column. The frog was without any external indications of the approaching metamorphosis. However, the section shows that the process is under way, for there is a dissolution of the muscle bands, a loosening up of the fibers, and, in the larger spaces, phagocytes are to be seen in the act of engulfing portions of the dissociated musculature. The important point to be noted, however, is that there has been a change in the condition of the musculature in regions where no phagocytes occur. This change involves what has

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been described as a "chromatolysis," but the dark masses running transversely over the muscle bands are depositions of fats bearing unsaturated fatty acids (oleic) which stain with the osmium tetroxid used in fixation. Willard and Guenther in unpublished investigations upon the micro-chemistry of degenerating muscle

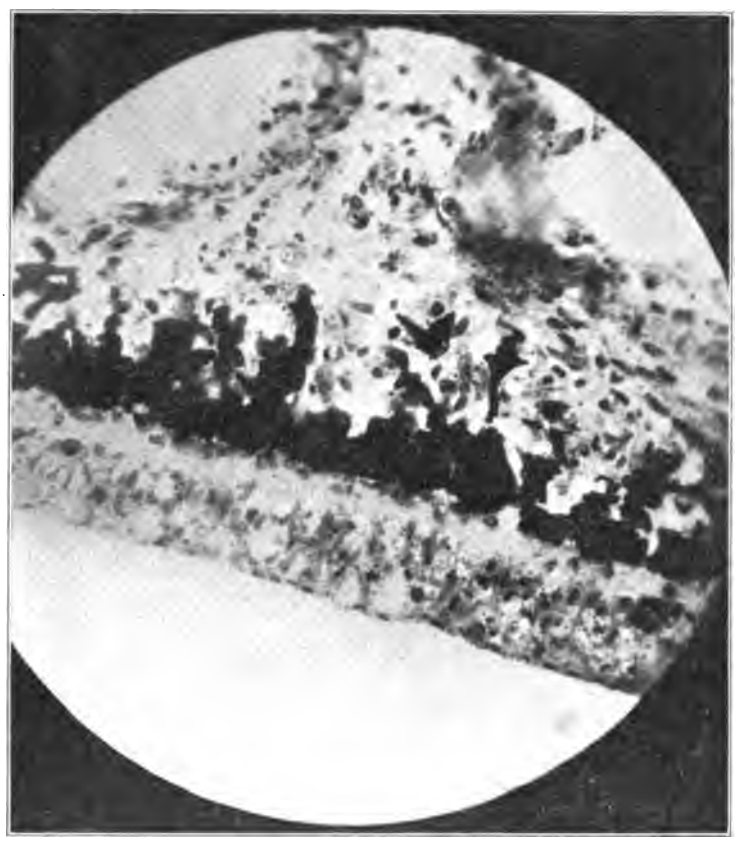

FIG. 2. Section of similar stage, same species, showing peripheral tissues. Not all of the intensely black areas are those of chromatolysis, for the osmium stains those neutral fats containing unsaturated fatty acids, such as oleic.

fibers find similar conditions in mammalian muscles permitted to degenerate after severing the innervation; a change in the distribution of neutral fats occurs involving the grouping of these compounds into masses, or droplets.[1] The significance in the present

[1] There is no evidence of an infiltration of fat, but rather of a simple process of accumulation of fats already present in diffused form.

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instance is that whatever the process means, it is precursory to phagocytosis.

In the second figure, the same mobilization of the lipoids is to be seen in the layer immediately proximal to the epidermis. This does not appear in control organisms where the size of the individual together with the character of the cellular elements in the muscles and elsewhere negate the advent of metamorphosis. Accompanying the so-called but misinterpreted "chromatolysis" is a vacuolization of the tissues, an edema recognizable in the translucence of the tail when viewed in the individual intact and alive. This edema formation is to be seen in the second figure lying directly distally to the dark area. The vacuoles seen in the epidermis, however, are normal skin glands. Regarding, now, the question as to whether phagocytosis is a factor here, it must be admitted that there is nothing to indicate the presence of these agents. It is difficult to present this testimony in a black-and-white photomicrograph, but sufficient detail is given to insure that at least the process is not *pari passu* with the extent of edema formation and of lipoid mobilization. Perusal of the microscopic sections themselves makes it evident that no phagocytosis is operative in these tissues, although there have been some changes indicative of metamorphosis.

From histological evidence, then, we must accept the belief of Looss and others supporting him that phagocytosis as a primary event is not a fact, but that its participation in metamorphosis is a later and secondary event.

In passing, it is of interest to compare the histological pictures given by preparations of Zenker's or hyaline degeneration and those given by the normal atrophy of the larval frog's tail. The writer was first struck with this coincidence in examining the photomicrographs of Steiner's (12) article on dermatomyositis. From histological evidence and from physiological considerations it is probable that the natural atrophy of the metamorphosing tadpole is reflected in the pathological atrophy in cases of progressive muscle atrophy.

From the evidence thus far submitted it is probable:

1. That an event precursory to the appearance of phagocytes in the affected areas occurs.

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2. That phagocytosis contrary to being a primary agent in effecting the dissolution of the tissues of the tail is of secondary importance.

Biochemical Evidence.

During the last few years, a more complete understanding of the softening of tissues in the living and in the dead organism without the participation of microörganisms has been obtained through the work of Bradley (13) and others. There is a more or less definite relation between reaction of medium and the process known variously as self-digestion, maceration, auto-digestion, autolysis, etc., wherein tissue enzymes resembling in a general way the enzymes of the alimentary tract operate in the tissues themselves, causing autolytic processes. This relation of reaction is such that when the average normal alkalinity of the tissues falls below a certain point, autolysis begins. In the terminology of physical chemistry, when the number of hydroxyl groups or ions (OH) approach (molecular weight in hydrogen equivalent)/(10,000,000) or $N/(10)^7$, which is neutrality for the ions involved, then either the proteolytic enzymes of autolysis are activated, or the reaction causes a change in the proteins rendering them digestible by the enzymes (14). A distinctly acid reaction, that is, where the concentration of OH ions is less than 1/10,000,000 normal, or, in the terminology of Sörensen which is being universally adopted, where $Ph < 7$[1], autolysis proceeds rapidly.

Up until the present time, the hydrogen ion concentration of the blood of the larval frog has not been accurately determined, a point to which the writer means to direct attention when material is available.[2] It is desirable to determine whether there

[1] The expression *P*h is borrowed from the principles of electromotive force, where the "potential" difference is measured on a potentiometer between a known concentration of hydrogen or hydroxyl ions and the unknown solution whose concentration in hydrogen or hydroxyl ions is required. The expression Ph signifies that the potential refers to hydrogen (h). Its numerical value is the same as the expression $N/(10)^a$, where a is a number greater than 7 if the solution in question is alkaline, or less than 7 if the solution is acid. Inasmuch as $N/(10)^a$ is the same as $N(10)^{-a}$, the expression *P*h means "minus the logarithm of concentration."

[2] Only the larger species of frogs can be utilized for these determinations. The arva of *Rana catesbiana* is of sufficient size to give 2 c.c. of blood for experimental

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is any change in alkalinity coördinate with atrophy. In the case of atrophy of the kidney tissue of the mammal where autolysis can be demonstrated, the writer and colaborator (15) have found that there is a rapid loss of alkalinity when autolysis sets in, while a true acidity develops later. If, in the larval frog, autolysis is *vera causa* for the process of atrophy, we should expect to find a low alkalinity or actual acidity of the body fluids of the tail during atrophy.

If autolysis actually produces atrophy of the larval frog's organs, we should expect to find a difference in the quantities of end products of protein digestion between the normal, non-metamorphosing tail and that which is atrophying. However, the practical demonstration could not be considered especially easy since the work of Van Slyke and others upon mammals has shown that these end products, the lower polypeptids and amino-acids, are rapidly withdrawn from the blood by the various tissues. Even after a full meal, until the micro-methods of Folin, Van Slyke and others were brought into play, amino-acids in the blood could not be satisfactorily demonstrated. *A fortiori*, in the atrophying frog, large differences in amino-nitrogen in the two cases in question are not to be expected. Another point should be recalled in this connection: Muscle, of the various tissues, undergoes autolysis slowest, with the exception of nervous tissue.

The following experiment was designed to show whether, qualitatively, a difference in concentration of amino-acids could be observed between normal and atrophying tissues.[1]

Experiment.—The tail of a larva of the common frog, *Rana areolata*, was used. The larva selected had small posterior legs, anterior ones still beneath the integument and histological sections of similar stages of *Rana catesbiana* larvæ failed to show evidence of metamorphosis. The tissue was weighed to 0.5 gram wet weight, chipped fine and transferred to a Schleicher and Schüll No. 579

purposes, which is practically the lower limit for work such as the determination of acidity where at least a half cubic centimeter of serum is desirable.

[1] The writer reported, earlier (16) upon the concentration of amino-acids in the tissues of the larval frog, where direct determination by the method of Van Slyke was attempted; from further work it is evident that this evidence is of little value since the calculated amounts of amino-acid would be extremely small.

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protein impermeable dialyzing thimble in which 1 c.c. of blood serum, free from hemoglobin, plus 2 c.c. of 0.7 per cent. NaCl solution had been placed. The thimble was suspended in 20 c.c. of distilled water, toluene added to both liquids within and without the thimble and placed in a thermostat at 30° C. over night. Similarly, tails from atrophying individuals were removed, and treated as the normal tail was prepared. At the end of 16 hours, the dialyzate in 2 c.c. amounts was boiled with three drops of 1 per cent. solution of "Ninhydrin" for one minute. The control (non-atrophying, normal) gave negative results while the atrophying tissue gave positive. Several duplicates gave similar results.

In interpreting this experiment, it may be urged that atrophying tissue digests more rapidly than normal tissue. To determine this point the following experiment was performed.

Experiment.—Sixty grams, wet weight, obtained by using the whole body of several non-metamorphosing individuals were ground in sand and made up to a 25 per cent. digest with toluene water. A similar digest was prepared from involuting larvæ. The preparations were incubated for five days at 37° C. Twenty-five cubic centimeter aliquots of the digests were pipetted into 50 c.c. tannic acid solution which was left to precipitate over night. The preparation was diluted to 100 c.c. filtered and 50 c.c. aliquots were examined by the Kjeldahl process for total non-protein nitrogen. Duplicates gave the following results:

Control	1.07 gm. protein per 60 gm. tissue.
Absorbing	1.68 " " " " " "

For the Van Slyke gasometric method, tannic acid could not be used to advantage as a protein precipitant; 50 c.c. of boiling saturated solution of potassium sulphate was made; otherwise the procedure was as before:

Control	110 mm. NH_2 nitrogen per 60 gm. tissue.
Absorbing	76 " " " " " " "

The formol-titration method gave:

Control	117 mm. NH_2 nitrogen per 60 gm. tissue.
Absorbing	95 " " " " " " "

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Obviously, there is no acceleration, but rather an apparent inhibition, which is not a true inhibition of digestion rate, but is due to the fact that autolysis has proceeded in these tissues before the death of the larva, the products being withdrawn by the circulation, leaving only the slowly autolyzing tissues for this experiment. The apparent increase of 1.68 g. over 1.07 g. in the case of the non-protein nitrogen figure, is insignificant, while the difference in non-protein and amino nitrogen is due to peptones and other polypeptids.

The tissues, then, are not in more favorable condition for self-digestion in the case of atrophying than in normal instances.

The question arises as to whether the enzyme content varies in the two cases. This is improbable. Loevenhart (17) has shown that in liver atrophy in mammals, an ester splitting enzyme, esterase, is not increased over that of the normal liver. Bradley (18), again, has shown that in the mammary gland there is no increase in lipase during lactation over the resting condition of the gland. Finally, the writer (19) has interpreted his experiments upon atrophy similarly. However, the following experiment seems to demand that, if it is true that the atrophying tissues themselves do not digest faster *in vitro* than non-atrophying masses, there is something in the enzyme relations in the two cases which is different.

Experiment.—Four tenths of a gram of tissue, wet weight, from a non-involuting larva were placed in 3 c.c. of serum from an adult frog. Toluene was added. A control consisting of 0.4 c. of involuting material was made. Digestion was permitted at 30° C. for 24 hours. Each preparation was then diluted to 5 c.c. of fluid with distilled water. 2.5 c.c. aliquots were examined for amino-nitrogen by the gasometric method of Van Slyke:

Control.....0.63 mm. NH_2 nitrogen per 0.4 g. tissue.
Absorbing.....1.19 " " " " " " "

Here, the differences are approximately 1:2 and inasmuch as we have seen that there is probably more autolyzable material taken from the involuting tissue, which has undergone a certain degree of self-digestion or atrophy (compare the preceding experiment), leaving less to digest in artificial autolysis experiments,

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the difference is significant. There are two substrates present in this system, namely, blood-serum proteins and secondly the tissue from the tail. The conclusion is warranted that there is a difference in enzyme content between the atrophying and the normal tissue. Granting that the serum proteins are more readily digested by the enzymes of the tail issue, we are still driven to assume that the enzymes are different in the two cases.

We have seen, then, that correlative with histological differences, there are enzyme differences in the case of atrophying and non-atrophying material.

It is possible that the experiments described in the preceding paragraphs do not bear critically upon the problem as to what induces atrophy of the metamorphosing larva. It may be urged that phagocytes could be operative in each of these cases. To assume this, it would be necessary to demonstrate that within the relatively short period of 24 hours, phagocytes, working *in vitro*, could perform the task of digesting the tissue in the amounts given. We should at once seek to answer the question as to whether a marked increase of phagocytes is to be observed in atrophying material over the normal. The writer (20) has made differential counts of the blood cells of normal and involuting individuals with the result that no increase commensurate with the difference in digesting power in the two cases exists. That there is a concentration of phagocytes (polynuclear leucocytes) in the affected areas is evident from Fig. 1, given above, and also from the work of Mercier; the actual number, however, remains the same.

The Causes Inducing Atrophy.

Mammalian experiments at the hands of Martin Jacoby and many other investigators have shown that atrophy involving autolysis is induced when the blood supply is interfered with. It is not necessary to occlude the supply directly; interruption of the blood supply will serve to cause atrophy. Bataillon (21) found that in the frog, the development of the pygostyle caused a change in the distribution of the blood supply throughout the tail. This does not involve complete occlusion, for Mercier found that, comparatively late in metamorphosis, phagocytes bearing carmin granules picked up from the dorsal lymph sac into which

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Mercier had injected the grains, appeared in the muscle masses undergoing atrophy.

Granting that this explanation is adequate, we may seek the more immediate factor involved. The first suggestion may be that oxygen inhibits the activity of the autolyzing enzymes and that when the blood supply is interfered with, the inhibiting action of oxygen is thrown off, permitting autolysis. The experiments of the writer (22) speak decidedly against this theory of inhibition of autolysis by oxygen.[1]

The known relation between reaction of medium and autolysis, of which we have spoken earlier in the present paper is sufficient to afford an adequate explanation of what takes place at the inception of autolysis. Partial interruption of the blood supply everywhere results in an accumulation of carbon dioxid. The "buffer" value of the blood in alkalinity is soon neutralized and an actual acidity of the blood results ($Ph<7$). Perhaps, likewise, as in starvation, acids other than carbonic acid enter the blood stream, such as those of incomplete oxidation—the so-called acids of acidosis, ketonic acids, beta-hydroxybutyric acid, aceto-acetic acid and the keton aceton. Even in the total exclusion of respiratory oxygen, intramolecular oxidation occurs, giving rise to carbon dioxid and to the acids of acidosis. This the writer takes to be the *modus operandi* of atrophy in the larval frog.

With regard to oxygen, the following experiment of the writer bears:

Experiment.—Several tall glass cylinders, 45 cm. high and 5 cm. in diameter were filled with water and into each was introduced a single larva which gave no evidence of metamorphosis. The larvæ were fed algæ, but owing to the darkened space in which the experiment was conducted, photosynthesis, giving rise to oxygen, did not occur. The jars were left for several weeks, during which time the larvæ grew, but none of them exhibited any tendency towards metamorphosis. Controls in finger-bowls with oxygenated water metamorphosed.

[1] The recent papers by Burge in the *American Journal of Physiology* contain the assumption of oxygen inhibition, but there is no experimental data to substantiate the theory.

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Experiment.—Tap water was boiled and cooled in hermetically sealed jars. Finger-bowls were filled with the water and larvæ introduced. There was a high mortality amongst the larvæ, but a few were kept for two weeks, during which time no metamorphosis occurred.

Experiment.—The dorsal nerve cord together with the aorta was ligated with silk thread, at the base of the tail. Again, the mortality was high, the open cut being attacked by *Saprolegnia* and water moulds, wild yeasts, etc. No acceleration in metamorphosis was observed.

Lack of oxygen, or a marked reduction in its amount does not induce autolysis in the larvæ. It must be admitted, however, that the negative results of the last experiment do not bear out the theory that interference in the blood supply induces atrophy. Lateral circulation, however, may have been a factor.

Wintrebert (23), seeking the cause of atrophy in the larval frog, suggested that an internal secretion induces metamorphosis. The following experiment was directed towards the obtaining of evidence upon this point:

Experiment.—Five normal larvæ were isolated in large petri dishes. Serum was injected into the dorsal lymph sacs of these larvæ, the serum having been obtained either from the blood of involuting larvæ or from the expressed juices of the tail. No attempt to keep the preparation sterile was made owing to the obviously impossible nature of the task. The results were negative as far as inducing metamorphosis.

Attention has been directed to the glands of internal secretion. Thus Babák (24) has studied the effects of injections of hypophysis and of other portions of the brain. Positive results were obtained which this writer interpreted as due to direct effects upon the nervous system rather than upon the tissues. Babák considers that there is a hormone-action involved in the sense of Bayliss and Starling. The thyroid has received a relative large amount of attention. Gudernatsch originally observed a precocious metamorphosis in the case of larvæ fed upon thyroid preparations and since his work, others have studied the problem from the biochemical aspect. David Marine (25), Lenhart, the writer (26) and recently, from a more general physiological

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aspect, Allen (27) have examined the relations of the thyroid and thyroid components upon metamorphosis. The writer found that an iodized amino-acid (3–5-di-iodo-tyrosin, $C_6H_5OHI_2\,CH_2.CH.NH_2.COOH$), induces metamorphosis in the frog's larvæ, this amino-acid being derived from the thyroid tissue by means of acid hydrolysis and hence being a normal component of the thyroid. These observations taken in connection with the interesting morphological studies of Allen give us a *rationale* of the rôle of the thyroid in inducing metamorphosis. When thyroid tissue or its components is fed to an organism, the general metabolism is increased as evidenced by the increased output of the end products of protein, carbohydrate and fat metabolism. When thyroid is removed from the economy of the organism, as in Allen's thyroidectomy experiments, metabolism is decreased; this is indicated by his further observations that reduced nourishment obtains the same results, that is, in both cases metamorphosis is suppressed or delayed. We may conclude, then, that metamorphosis involves, as one of its principal factors, heightened metabolism.

It is necessary to distinguish between heightened metabolism, which involves only *catabolism*, in Gudernatsch's experiments and the accelerated anabolism present in Allen's cases. In both instances, metabolism is heightened, but the current is down stream in the one case, leading to loss of storage materials and a piling up of materials in the form of fat, etc., in the second case. Now it is of interest to recall that according to Voegtlin and Strouse, the iodized amino-acid which was found to operate as whole thyroid tissue in inducing precocious metamorphosis, fails to replace the function of thyroid in those pathological cases where there is a deficiency in thyroid function. It would be of considerable interest to determine whether 3–5-di-iodo-tyrosin, thyreoglobulin or any of the chemical components of the intact thyroid tissue replaces the absent thyroid in cases of thyroidectomy in inducing metamorphosis, for wrapped up with the question is the important and fundamental one as to what relation the iodine has in the thryoid. If the iodized amino-acid should in itself, in the absence of the thyroid, induce metamorphosis, it would show that the theory that iodine stimulates an

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internal secretion in the thyroid gland, rather than functioning as hormone itself, is untenable. In either case it would clear up the apparent discrepancy in the findings of Voegtlin and Strouse and in those of the students of amphibian metamorphosis.

The Economy of the Process.

In the investigations upon the salmon conducted by Miescher and later by Noel Paton and others, it is evident that there is a high degree of economy involved in the process of metamorphosis of the muscles into gonad material. The question arises, is this true for the metamorphosis of the larval frog?

In the foregoing paragraphs, we have spoken of this process of metamorphosis as if it were confined to the regions of the tail, but the difficulty of studying the participation of the alimentary tract, gills, etc., in the process is not a small one and these investigations have been confined to the changes in the tail. One primary difficulty regarding the study of the alimentary tract is that at metamorphosis, preceding the shortening of that system, a large accumulation of fecal matter is expelled, containing the same chemical compounds which are utilized in studying the transformations of tissues. It is for this reason that the simple expedient of weighing the organism before and after metamorphosis to determine the loss, if any, of material, is not feasible, the fecal matter far overbalancing any amount of product of tissue change thrown to the outside.

A study was made of the excretions of the larvæ immediately after the expulsion of the fecal matter. Larvæ were isolated in distilled water in large petri dishes. After twelve hours, the water was filtered and estimations were made of the total nitrogen, amino-nitrogen, ammonia and urea. While the method was crude, owing to the impossibility of inhibiting the transformation of urea into ammonia and of the probable deaminization of the amino-acids by bacteria, there was no constant difference in the figures for non-metamorphosing and metamorphosing lavræ. Unless, then, there were extensive excretion of the products of the decomposition of the larval tissues, it is safe to conclude that the process is an economic one, the tissues of the larval organs contributing to the formation of those of the adult.

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Pointing to the correctness of this interpretation is the fact that during the development of the adult organs (legs, etc.) no food is taken by the larva. Storage of fat, etc., has taken place during the earlier period of larval existence, but the important thing is to remember that *protein is never stored as such in the animal organism.* The nitrogenous material for the legs, etc., developing during metamorphosis must be supplied from some source other than storage. The simplest explanation is to assume that it is derived from the larval organs.

Von Baer's Law and Larval Metamorphosis.

The frog has been taken as a classic example of the operations of the so-called Law of Von Baer, termed sometimes the Law of Recapitulation and at others that of Repetition. The question is, can the metamorphosis be explained wholly upon the assumption that physico-chemical events other than those concerned with heredity initiate the process?

Those who have experimented with the agents accelerating metamorphosis have found that these compounds are impotent except in the case of larvæ which have reached a certain period of their existence. In other words, we know of no agent which is operative regardless of the stage of development of the larvæ. We may suppress metamorphosis; we can not, or have not induced it in stages far removed from those in which it would normally occur. This indicates that a certain cycle of events, probably determined by heredity, are necessary before any stimulating agent is effective. The growth of the pygostyle according to Barfurth, initiates the process of atrophy in the tail, but what events stimulate the growth of the pygostyle are unknown. In some species, which do not metamorphose during the first summer, the larvæ are subjected to relatively the same stimuli from the environment during the second summer as during the first. It is improbable that external conditions determine the time of metamorphosis. Again, to offer the explanation that hormones or enzymes initiate metamorphosis leaves open the more potent question as to what determines the development of these compounds.

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CONCLUSIONS.

1. Autolysis is the primary factor in the physiology of atrophy in metamorphosis.

2. This process is probably induced by an acidosis in the tissues.

3. Adopting Barfurth's suggestion of the occlusion of the blood vessels at the base of the tail by the growth of the pygostyle, it is probable that carbon dioxid and acids of incomplete combustion accumulate and render the reaction of the blood less alkaline or more acid.

4. Phagocytosis is not a primary factor, as Metchnikoff believed, but a secondary one, the phagocytes being attracted by chemotaxis to the atrophying organs.

5. The process of metamorphosis is economic, the substances derived from the atrophying organs being utilized by the growing adult organs.

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STUDIES IN THE PHYSIOLOGY OF SPERMATOZOA.[1]

EDWIN J. COHN.

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[1] From the Marine Biological Laboratory, Woods Hole, Massachusetts, and the Hull Zoölogical Laboratory, The University of Chicago.

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I. Introduction.

The changes in the physiological condition of the spermatozoon, from the time it is extruded from the genitalia of the male until it "undergoes the transformation into a nucleus" (Loeb, J., 1913, p. 306) in the protoplasm of the egg are dependent in rate upon environmental conditions. The germ cells of most marine invertebrates are extruded into sea water, and fertilization of the egg by the sperm there follows. The environment, sea water—or sea water modified by the excretions of the egg or of the sperm—must therefore be studied in order to understand the variations in the physiological condition of spermatozoa that have often been observed.

This investigation had its beginning in an attempt to understand seemingly contradictory effects of sea water that had contained the eggs of the sea urchin,[1] *Arbacia punctulata*, upon the activity, the length of life and the "fertilizing power" of the spermatozoa of the same species. For interesting me in these phenomena, and for invaluable aid in this attempt at their solution, I am indebted to Dr. F. R. Lillie.

The experimental work was carried on during the summers of 1915 and 1916 at the Marine Biological Laboratory at Woods Hole, Massachusetts. During that time the behavior of the germ cells of other marine invertebrates were sufficiently observed to suggest that the relations that are hereafter reported for *Arbacia* are not highly specific.

Material and Methods.

The sperm of the sea urchin, *Arbacia punctulata*, are shed from the genital pores, if the peristome is cut, and the urchin placed aboral side down. The sperm may then be collected in a clean dry watch glass, and diluted to any concentration by the admixture of sea water. The concentration of the sperm suspension records the extent of dilution of the sperm. A one per cent. suspension is one in which one drop of sperm is added to 99 drops of sea water or of sea water that has suffered a definite modification.

The relative "fertilizing power" of sperm that had been sub-

[1] Such sea water is for convenience called egg water.

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jected to different environments was determined by adding such sperm in identical concentration to the ripe eggs of the same species in sea water at different times. The average number of eggs that subsequently developed was estimated by counting at least a hundred eggs at about the four cell stage.[1]

In tabulating the experiments that are reported (in Tables I., II. and III. the concentration of spermatozoa was the variable environmental condition) the concentration of spermatozoa in the suspension is recorded in a column at the extreme left. The variable environmental conditions that obtained in the different suspensions are recorded in a legend over the columns representing the percentage of eggs that were fertilized when a definite number of drops of the sperm suspension (the number of drops of sperm is reported either in the legend or in a column at the extreme right) were added to a constant quantity of eggs in a given amount of sea water at the intervals noted.

The effects of change in environmental condition upon sperm were under investigation. Since the influence of change in environmental condition upon eggs and upon sperm are not dissimilar (Loeb, J., 1913, Robertson, T. B., 1912) the variation in the physiological condition of eggs (Gemmill, 1900; Vernon, 1899; Loeb, J., 1913; Goldfarb, A. J., 1917) was eliminated by always inseminating in sea water.

For the same reason, the eggs of but one female were used in each series of inseminations. The fertilizing power of different sperm suspensions were in this way tested. The eggs were obtained by straining the cut up ovaries through cheese cloth into sea water, and subsequently washing the eggs by decanting the supernatant fluid. Eggs were never used after they had been in sea water for more than six hours. The forceps that were employed in removing the ovaries were never used for any other purpose. If a male had previously been opened, the hands and the scissors with which the peristome had been cut were rinsed in fresh water. A control of unfertilized eggs was always kept, but no contamination was ever observed.

The failure of sperm to fertilize ripe eggs may be employed as

[1] See Lillie, F. R., 1915; Fuchs, H. M., 1915, for a detailed description of this procedure

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an indication of death only if accompanied by some other observation such as the dissolution of the protoplasm that follows the death of spermatozoa. This is necessary, since a decrease in the activity of spermatozoa may also decrease their fertilizing power. But sperm that are non-motile can be reactivated, and until reactivation is no longer possible, sperm can not be considered dead.

The rate of movement of spermatozoa cannot, however, be observed with any degree of accuracy. While it is not difficult to distinguish between a very motile, a fairly motile and a non-motile sperm suspension, more delicate fluctuations in spermatozoön activity cannot easily be observed. As in other cells a delicate indicator of the degree of activity is afforded by the measurement of the oxygen consumption (Loeb and Wasteneys, 1912; Warburg, 1910) the heat production (Meyerhoff, 1911) or the carbon dioxide production of a sperm suspension. The carbon dioxide production of sperm suspensions of different concentration has been measured.

The activity of spermatozoa, as will presently appear, is effected by changes in either the temperature, the osmotic pressure or the hydrogen ion concentration of sea water. Since even the carbon dioxide produced by spermatozoa is sufficient appreciably to change the hydrogen ion concentration of a 0.005 per cent. suspension, since the activity of spermatozoa is a function of the hydrogen ion concentration, and since the fertilizing power of a sperm suspension is related to the activity of the spermatozoa, the careful control of the environment becomes a necessity.

Sea Water as Environment.

The environment of the spermatozoa of the sea urchin is sea water. A variable in sea water that is known to effect the activity of spermatozoa is the hydrogen ion concentration. The concentration by weight of hydrogen ions in sea water is approximately 0.00000001 N or 1×10^{-8} N (Palitzsch, Sven, 1912). In a neutral solution there are, by definition, as many hydrogen as hydroxyl ions. The concentration of hydrogen ions in a neutral solution is 1×10^{-7} N or 10×10^{-8} N. There are therefore in the neighborhood of ten times as many hydrogen ions

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in a neutral solution as there are in sea water and ten times as many hydroxyl ions in sea water as there are in a neutral solution. Since the acidity or the alkalinity of a solution is measured in terms of its concentration in hydrogen ions, sea water is appreciably alkaline. The hydrogen ion concentration is often expressed as the negative logarithm to the base 10. This is called the hydrogen potential (Ph) (Sörensen, S. P. L., 1909). The hydrogen potential of sea water is therefore Ph 8. During the months of July and August, 1916, the sea water at the Marine Biological Laboratory at Woods Hole only varied between Ph 7.95 and Ph 8.15.

The hydrogen ion concentration of sea water was measured by colorimetric comparison with solutions of borates and phosphates. These were always standardized with a concentration cell. The indicators phenolphthalein, naphtolphthalein and neutral red satisfactorily covered the range investigated. Corrections for the effect of salts upon these indicators have been determined (Sörenson & Palitszch, 1910, 1913; Palitszch, 1911) and were employed.

The hydrogen ion concentration of "sea water is fully determined by (1) the tension of carbonic acid, (2) the concentration of water, or salinity, and (3) the temperature. This relationship suggests a method of determining the carbon dioxide tension of sea water" (Henderson, L. J., and Cohn, E. J., 1916, p. 620), for the effect of salinity upon the hydrogen ion concentration has been found to be very small (Henderson, L. J., and Cohn, E. J., 1916; McClendon, Gault and Mulholland, 1917). The effect of the temperature has been determined (Henderson, L. J., and Cohn, E. J., 1916; McClendon, Gault and Mulholland, 1917), and the relation between the hydrogen ion concentration and the carbon dioxide tension of sea water at a temperature of 20° C. has been reported by Henderson & Cohn (1916); at a temperature of 30° C. by McClendon (1916 and 1917). The measurement of the carbon dioxide tension of sea water (that is the partial pressure of the gas that is in equilibrium with sea water containing a definite concentration of carbon dioxide) can therefore be made with great accuracy and great rapidity. The carbon dioxide tension is recorded in terms of the number of millimeters of

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mercury that represents the partial pressure of the gas in the atmosphere at a total pressure of 760 millimeters, and a temperature of 0° C.

The change in the carbon dioxide concentration of sea water is not proportional to the change in the carbon dioxide tension, for increase in the carbon dioxide tension is correlated with a change in the equilibrium between the normal carbonates and bicarbonates in sea water. It has been calculated (Henderson, L. J., and Cohn, E. J.) that the former are converted into the latter at exactly the tensions of carbon dioxide that obtain in the ocean. At tensions of carbon dioxide greater than these an increase in tension may, as a first approximation in determining the carbon dioxide concentration, be considered as an increase in free carbonic acid. A "conversion table" for determining the carbon dioxide concentration (or content) of sea water has been published by McClendon (1917). The measurements of the total carbon dioxide concentration upon which this "conversion table" is based are not reported. Exact data defining the relation between the hydrogen ion concentration and the carbon dioxide concentration of sea water are therefore still unknown.

II. Environmental Conditions that Affect the Activity of Spermatozoa.

That "all the phenomena connected with the origin and death of the spermatozoön seem to be in accordance with the view, that its motion is essential to its function" (Newport, G., 1853, p. 261) was the opinion of the early investigators of the rôle of the sperm in fertilization. Indeed so completely was the movement of the "spermatic animalcules" found to depend upon temperature (Spallanzani, quoted from Newport, 1851, p. 235) (Prevost and Dumas, 1824) (Newport, G., 1851); osmotic pressure (Koelliker, A., 1856) and hydrogen ion concentration (Koelliker, A., 1856) that the observed activity of these cells was for a time supposed to be due to Brownian movements.

"Es fällt somit die Theorie, die Bewegung der Samenfäden sei willkürliche thierische Bewegung, haltlos zusammen. Welche physikalischen Kräfte aber dieses Phänomen erzeugen mögen, ist noch völlig dunkel. Ja wir können noch nicht einmal mit Bestimmtheit behaupten, obwohl diess wahrscheinlich ist, dass

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die Samenfäden auch im Organismus, im Hoden oder in den weiblichen Genitalien sich bewegen, es kann Niemand mit Bestimmtheit widerlegen, dass nicht etwa diese Bewegungen erst in den aus dem Organismus entfernten Objecten unter dem Mikroskop, als ein Analogon der Brown'schen Molecularbewegung entstehen, sei es durch Verdunstung oder irgend eine andere physikalische Ursache. Es ist mehr als wahrscheinlich, dass die Bewegungen wenigstens in einer physikalischen Wechselwirkung zwischen Flüssigkeit und Samenfäden begründet sind, wofür schon die ausserordentliche Abhängigkeit der Bewegungen von der Concentration und Beschaffenheit der Flüssigkeit, ferner vor Allem die Abänderung der Bewegungsacte durch Zusatz von Wasser, die Abhängigkeit der Art der Bewegung von der Form der Samenfäden der verschiedenen Thiere spricht, Umstände, welche auch auf andere Weise als durch einfache Adhäsionsverhältnisse, Vermehrung und Verminderung des Widerstandes zu wirken scheinen" (Koelliker, A., 1856, p. 202. Quotation from Funke im Lehrbuch der Physiol. von Günther, Bd. II., Abth. IV., 1853, p. 1027).

Dissenting from this position Newport "regarded this motion as being only the visible indication of a peculiar force, or form of vitality, in the impregnating agent, the spermatozoön, by which it is destined to arrive at, and is to expend on the object to be fecundated, and the effect of which is to strengthen, to augment, and possibly also to modify the nature of the formative changes, which are going on in the yet unimpregnated egg, per se; but which will subside, and soon entirely cease, if not reinforced through the agency of the spermatozoön" (Newport, G., 1853, p. 260). And again: "Whatever be the relation of this motion to its peculiar faculty, it is evident that motion is intimately associated with, and dependent on, its material composition and structural development" (Newport, G., 1853, p. 261).

Temperature.

The environmental conditions that effect the behavior of the spermatozoön were therefore abundantly and carefully observed by early investigators. "Spallanzani found that the fluid of the fœtid terrestrial toad (*Bufo calamita?*) at a high temperature of

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the season, 70° F. to 73° F., at which this species spawns in Italy, had lost its fecundatory influence at the end of six hours; but that in the temperature of an ice house, 40° F., it retained its efficacy for 25 hours" (Newport, G., 1853, p. 235). Observations of this kind were repeated by Prevost and Dumas (1824) and Newport (1851 and 1853) who wrote: "The general conclusion which seems to be deducible from a comparison of the observations of Spallanzani and of Prevost and Dumas, with those by myself, in regard to the tail-less Amphibia, is, that . . . the vitality of the spermatozoön, and the duration of its fecundatory power, are in a ratio inverse to that of an increase of temperature in the surrounding medium" (Newport, G., 1853, p. 237).

Hydrogen Ion Concentration.

Nor was temperature the only environmental factor that was known to effect the physiological condition of the spermatozoön. In a classic paper, "Physiologische Studien über die Samenflüssigkeit" (Koelliker, A., 1856), Koelliker demonstrated that the effect of substances supposedly "harmful" to the activity of the sperm, disappeared if the solutions were first made isotonic and isohydric with the suspension. He understood that a slightly acid solution might inhibit the activity of the sperm without killing them, and that reactivation followed upon neutralization of the acidity. He observed that if KOH, Na_2HPO_4 or blood were added to suspensions of paralyzed spermatozoa motility was recovered.

That the degree of activity is a function of the hydrogen ion concentration of the sperm suspension has been confirmed by subsequent investigations. In 1907 Günther (Günther, G., 1907) showed that not only could sperm be reactivated by a decrease in the hydrogen ion concentration, but also that they could be inactivated by an increase in the hydrogen ion concentration. He noticed that if a weak electric current is passed through a mammalian sperm suspension the sperm congregate at the positive pole and are there inactive. The hydrogen ion concentration is greatest at the positive pole. If the current is reversed sperm that have been inactive at the positive pole recover their activity and accumulate at the other

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end of the suspension; now become the positive pole. There they are again inactive. In the more alkaline part of the suspension the sperm are extremely active. Frog sperm (Lillie, R. S., 1903) and Echinid sperm (Gray, J., 1915) behave in a similar manner. This phenomenon does not occur if sperm are first inactivated by an increase in the hydrogen ion concentration of the suspension (Gray, J., 1915).

Oxygen and Carbon Dioxide Concentration.

Similar in kind is the observation of Buller (Buller, A. H., 1902) that when a bubble of oxygen is incorporated in a suspension of *Echinus* sperm those in the immediate vicinity of the oxygen remain active after the sperm at a greater distance "have all come to rest from want of oxygen." Between the active sperm and the inactive sperm there is a zone "in which there are comparatively very few spermatozoa." The active sperm gradually traverse the clear zone "and collect on the inner edge of the zone" (of inactive sperm) "upon reaching which they cease to move. A ring of thickly placed, dead spermatozoa thus arises" (Buller, A. H., 1902, p. 158). The spermatozoa were not "dead" but merely inactive in a region of low oxygen and of high carbon dioxide concentration.

F. R. Lillie has observed the converse phenomenon. He injected a drop of sea water with a carbon dioxide concentration of approximately 1 per cent. into a suspension of *Nereis* sperm (the sperm of *Arbacia* are not so sensitive to carbon dioxide but "the reactions of *Arbacia* spermatozoa are essentially the same in principle as those of *Nereis*") (Lillie, F. R., 1913, p. 546) and noticed that a ring of sperm is formed at a definite concentration of carbonic acid. This ring is separated by a clear zone from the active sperm in the rest of the suspension. "If the external edge of the clear zone be carefully observed, the spermatozoa can be seen to detach themselves one by one from the general suspension and pass straight over to the ring" (Lillie, F. R., 1913, p. 535).

It is apparent that increase in the hydrogen ion or in the carbon dioxide concentration or decrease in the oxygen concentration[1] decreases the activity of spermatozoa, while de-

[1] The observations of Drzewina, A., and Bohn, G. (Drzewina, A., and Bohn, G., 1912) upon the effects of lack of oxygen upon the length of life of spermatozoa will be considered in another place.

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crease in the hydrogen ion or carbon dioxide concentration or increase in the oxygen concentration increases the activity of spermatozoa. Three explanations of the configuration that appears when spermatozoa are subjected to such a gradient in carbon dioxide as has been described have been suggested; (*a*) that spermatozoa are activated in certain concentrations of carbon dioxide; (*b*) that spermatozoa are positively chemotactic or chemotropic to carbon dioxide; (*c*) that the accumulation of spermatozoa at a certain concentration of carbon dioxide is brought about by their inactivity in that concentration of carbon dioxide.

While it is not inconceivable that spermatozoa are activated in certain concentrations of carbon dioxide, no evidence of such a primary stimulation of spermatozoa has ever been observed or reported.

The circumstances which led to the formulation of the second hypothesis are quite intricate and will require an historical introduction if the problem is to be understood. Ever since Pfeffer "demonstrated the importance of the part played by chemotactic stimuli in causing the spermatozoa of liverworts, mosses, ferns, etc., to approach the oöspheres" (quoted from Buller, A. H., 1902, p. 145) biologists have tacitly assumed or attempted to demonstrate that this chemotactic phenomenon is general in fertilization not only in plants[1] but in animals. In 1895 Bergh suggested that "the spermatozoa collect around the ripe eggs, probably attracted by a special substance "(quoted from Buller, A. H., 1902, p. 146). Three years later Massart (Massart, J., 1888) demonstrated that the spermatozoa of the frog were positively thigmotactic to glass. He was, however, unable to demonstrate chemotaxis. This observation had previously been made by Dewitz (Dewitz, J., 1886). Massart also maintained that spermatozoa were positively thigmotactic to agar and gell (Massart, J., 1888) especially that of the egg (Massart, J., 1889).

[1] There is some doubt that chemotaxis is a general phenomenon in the fertilization of plants. To a recent study of the "Physiology of Fucus Spermatozoids" the following summary is appended. "Using the Pfeffer capillary tube method of determining chemotaxy, it was found that certain acids cause collection of *Fucus* spermatozoids. It is suggested that this may be explained as due to toxicity and not chemotaxy" (Robbins, W. J., 1916, p. 130).

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In 1900 Buller observed the "agglutination" or "cluster formation" of *Arbacia* sperm in water that had contained eggs of the same species and suggested that "a tactile stimulus appears to play a part in the phenomenon" (Buller, A. H., 1900, p. 387). As the "aggregation" of sperm by carbon dioxide has been supposed to be due to chemotaxis, so also has the "agglutination" of sperm by "egg water" (de Meyer, J., 1911, Lillie, F. R., 1913; Glaser, O., 1914). The phenomenon of "agglutination" will be discussed in another place. The configuration of *Arbacia* sperm in "agglutination" and of *Nereis* sperm in "aggregation" is not dissimilar (Lillie, F. R., 1913).

In 1902 Buller described the accumulation of inactive sperm in an oxygen gradient. This has been characterized as the converse of Lillie's "ring formation" in a gradient of carbon dioxide (p. 12). Buller explained the phenomenon as the result of the differential activity of spermatozoa in the gradient. He was, however, unable to account for the "clear zone" that occurs in the configuration. In consequence subsequent workers have had recourse to the accessory hypotheses that have been discussed. While there is no a priori reason for believing either that spermatozoa are not activated in certain concentrations of carbon dioxide or that they are not chemotactic to carbon dioxide, it should be pointed out that no positive evidence for either assumption has ever been demonstrated.[1]

III. The Relation between the Activity and the Longevity of Spermatozoa.

Increase in the activity of spermatozoa leads to a decrease in the length of time during which spermatozoa exhibit activity. This was observed by Koelliker, who remarked, regarding the effect of the alkali salts of carbonic acid, that they behaved much as the caustic alkalies in that: "Sie erregen die Samenfäden lebhaft, doch dauert deren Bewegung nicht lange" (Koelliker, A., 1856, p. 239). Koelliker emphasized that this activation oc-

[1] "Dewitz, Buller, and the writer have vainly tried to prove the existence of a positive chemotropism of spermatozoa to eggs of the same species" (Loeb, J., 1916, p. 93).

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curred only in weakly alkaline solution, since sperm were "injured" in more concentrated alkaline solution.

From these and other observations Koelliker came to believe that the nourishment and therefore the length of life of the spermatozoön after it is liberated from the testes is dependent only upon the material of which it is constituted. "Eher wäre daran zu denken, ob nicht vielleicht die Körper der Samenfäden sich zu den Fäden selbst verhalten, wie eine Zelle zu ihren Wimperhaaren, und dieselben aus dem in ihnen enthaltenen reichlichern Material ernähren, eine Vermuthung, die jetzt, wo ich zeigen kann, dass die Samenfäden aus den Kernen der Samenzellen sich bilden, wohl ausgesprochen werden darf. Zu erforschen ist auch noch, ob die Samenfäden bei ihren Bewegungen elektrische Ströme entwickeln, und ob sie nicht, so lange sie sich bewegen, CO_2 abgeben, während sie O aufnehmen, Verhältnisse, über die ich vielleicht später berichten kann" (Koelliker, A., 1856, p. 245).

On the basis of observations of a quite similar kind Gemmill (1900) came to exactly the opposite conclusion. He observed that "the term of vitality of spermatozoa varies according to the degree of their admixture with sea water" (Gemmill, J. F., 1900, p. 170) and correctly concluded that "mixing with seawater stimulates the activity of movement of the spermatozoa, which become the more active the better they are mixed with pure sea water. Under these circumstances, their store of energy will be the sooner exhausted" (Gemmill, J. F. 1900, pl. 169). Gemmill was of the opinion, however, that if sperm lived longer in more concentrated suspensions it was because they there received more nourishment, for he goes on to say: "On comparing the movements of spermatozoa in different mixtures, one finds that the difference of activity is not sufficiently marked to account for the very early loss of vitality of spermatozoa in the weaker mixtures simply in terms of exhaustion of energy. I am inclined to believe that the other factor above noted, namely, the dilution of the nutritive medium by the addition of sea water, is the more important cause. An interesting sidelight on this question is afforded by some facts which will be given later regarding the keeping alive of spermatozoa by artificial nutri-

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tion" (Gemmill, J. F., 1900, p. 171).[1] An experiment similar to those performed by Gemmill is reported (Table I., Experiment 28). The "term of vitality" of spermatozoa was determined by testing their fertilizing power. As in Gemmill's experiments the sperm in the most concentrated suspensions lived for the longest time.

TABLE I.

EXPERIMENT 28.

The Length of Life, as Measured by the Fertilizing Power, of Sperm Suspensions of Different Concentration.[2]

Date.	Time of Insemination.	Age of Sperm.		1	2	3	4
				Concentration of Sperm Suspensions.			
				4%.	1%.	0.5%.	0.25%.
		Hrs.	Min.	Percentage of Eggs Fertilized when 1 Drop of Sperm Added to 5 Drops of Eggs in 10 c.c. of Sea Water at Intervals as Noted.			
July 25..	12:20 A.M.	0	0				
" " ..	2:30 P.M.	14	10	100	98	67	10
" 26..	12:00 M.	23	40	100	98	15	0
" 27..	11:20 A.M.	47	00	100	0	0	0
" 28..	12:15 P.M.	71	55	98	0		
" 29..	8:20 A.M.	92	00	85			

The measurement of the total carbon dioxide production in sperm suspensions of different concentrations, which will now be reported, has however made untenable the position of Gemmill that the "exhaustion of energy" is not sufficient to account for the "loss of vitality of spermatozoa."

The Measurement of the Total Carbon Dioxide Production of Sperm Suspensions of Different Concentration.

The hydrogen potential of sperm suspensions of varying concentration was measured as a function of time.

In measuring the hydrogen potential of sperm suspensions

[1] The so-called "artificial nutrition" was brought about by adding beef broth to sea water. This increases the hydrogen ion concentration of the suspension and decreases the activity of spermatozoa. The evidence for these statements will be found in another place in this paper.

[2] The sea water in this experiment was sterilized in order to prevent the bacterial contamination which otherwise occurs when sperm suspensions are kept for so long a time (Gorham, F. R., and Tower, R. W., 1902). Erlenmeyer flasks were used in the experiment. On the third day suspension 1 was still relatively free from bacteria. The spermatozoa appeared healthy and were not motile.

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no such accuracy can be attained as in the measurement of the hydrogen potential of sea water, since the cloudiness of the more concentrated suspensions makes the colorimetric determination difficult and since the intensity of the indicators changes more rapidly in the presence of sperm. This is probably due to the

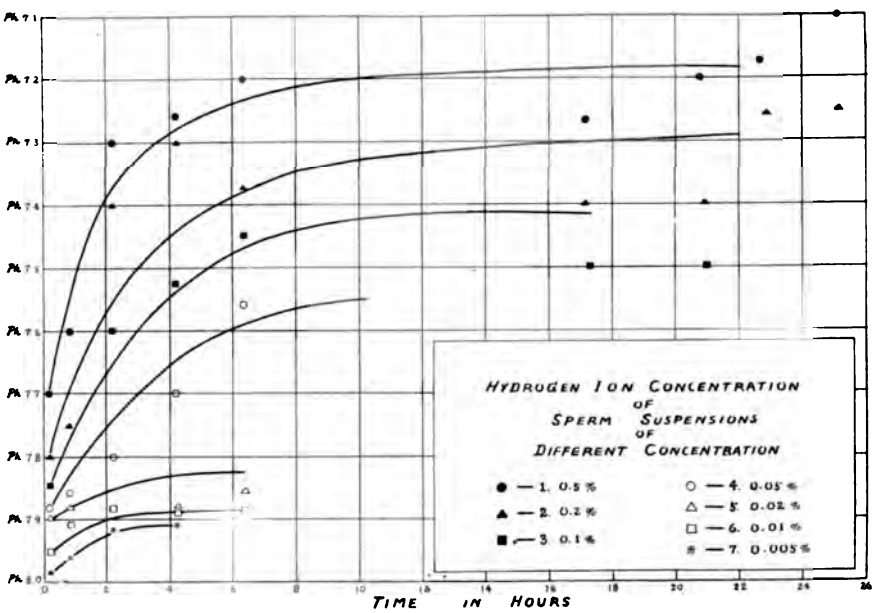

Diagram I.

penetration of the indicators into the cells and observations suggest that this depends upon the physiological condition of the sperm. These effects can, however, be prevented from interfering with the measurement of the hydrogen potential of the suspensions if the measurements are made immediately after the indicators are added. If these precautions are observed, a sufficient accuracy for biological purposes can be attained.

The data obtained in this way are in Table II. and are graphically represented in Diagram I. The ordinate represents the hydrogen potential of the suspensions; the abscissa, the time in hours. The concentration of sperm in each suspension is recorded in the accompanying tabulation. Since the increase in hydrogen ion concentration is due to the carbon dioxide produced

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by the spermatozoa, and since the rate of carbon dioxide production is, in turn, a function of the hydrogen ion concentration of the suspension, in all measurable concentrations the carbon dioxide production of sperm suspensions falls off with time.

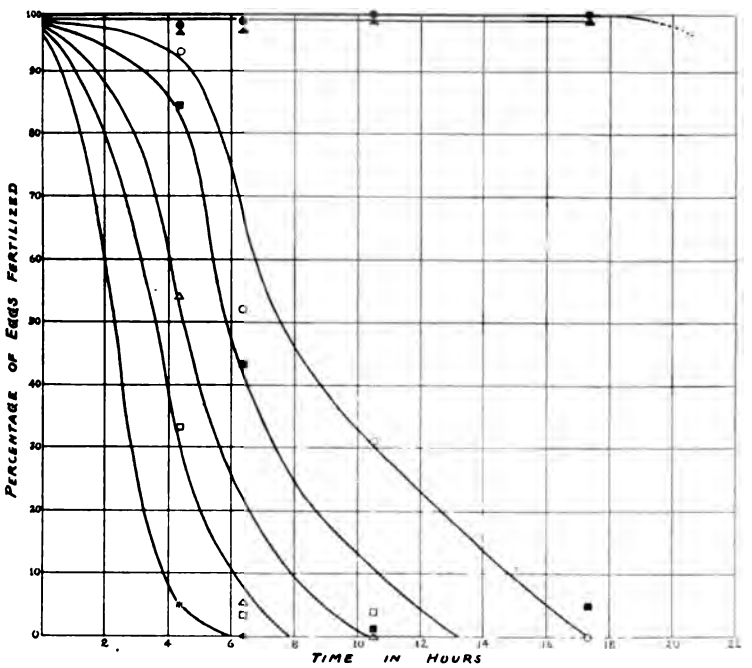

DIAGRAM II.

"The term of vitality of spermatozoa varies according to the degree of their admixture with sea water" (Gemmill, J. F., 1900, p. 170). In the most concentrated suspensions the spermatozoa live for the longest time. The length of life of the different suspensions as computed from the per cent. of eggs that were fertilized by identical concentrations of sperm under identical experimental conditions is recorded in Table III. and graphically represented in Diagram II.

The concentration of carbon dioxide, like the tension of carbon dioxide with which it is in equilibrium, in any one sample of sea water at any one temperature is completely determined by the hydrogen potential. As an approximation, sufficient for the

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calculation of the carbon dioxide produced by spermatozoa in the above experiment, an increase in carbon dioxide tension (as measured by change in hydrogen potential) may be considered as an increase in free carbonic acid.

Table II.

Hydrogen Potentials of Sperm Suspensions.

Age of Sperm Suspensions.		1	2	3	4	5	6	7
		Concentration of the Sperm Suspensions.						
		0.5%.	0.2%.	0.1%.	0.05%.	0.02%.	0.01%.	0.005%.
Hours.	Minutes	Hydrogen Potentials of Sperm Suspensions.						
	5	7.70	7.80	7.85	7.88	7.90	7.95	7.98
	50	7.60	7.75	7.80	7.86	7.88	7.91	7.96
2	20	7.30	7.40	7.60	7.80		7.88	7.92
4	20	7.26	7.30	7.53	7.70	7.87	7.87	7.91
6	20	7.20	7.37	7.45	7.56	7.86	7.88	7.95
17	20	7.26	7.40	7.50	7.74	7.90	7.98	8.04
20	50	7.20	7.40	7.50				
22	50	7.17	7.26	7.40	(7.74)	(7.88)	(7.91)	(7.98)
24	50	7.10	7.26	7.40				

Table III.

Length of Life of Sperm Suspensions.

Age of Sperm Suspensions.		1	2	3	4	5	6	7
		Concentration of the Sperm Suspensions.						
		0.5%.	0.2%.	0.1%.	0.05%.	0.02%.	0.01%.	0.005%.
Hours.	Minutes.	Percentage of Eggs that Were Fertilized in Sea Water by Identical Concentrations of Sperm.						
4	20	98	98	85	94	54	34	6
6	20	99	97	43	52	6	4	0
10	20	100	100	2	31	0	4	7
17	20	100	100	5	0	0	0	0
		Approximate length of life of spermatozoa as computed from their failure longer to fertilize eggs of the same species.						
		17+	17+	17+	10+	6+	6+	4+

The hydrogen potential of sperm suspensions of different concentrations at different times has been reported (Table II.). The total carbon dioxide production of each sperm suspension is equal to the difference between the carbon dioxide concentration of the sea water at the beginning of the experiment and at the time of the death of spermatozoa. If the total carbon dioxide

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production is divided by the concentration of spermatozoa in the suspension, the carbon dioxide production per unit concentration of sperm is obtained.

The relative carbon dioxide production of sperm suspensions of different concentrations is reported in Table IV.

TABLE IV.

TOTAL CARBON DIOXIDE PRODUCTION OF SPERM SUSPENSIONS.

No.	Sperm Concentration.	Calculation of the Relative Carbon Dioxide Production per Unit Concentration of Sperm.	Approximate Length of Life of Spermatoza.
1	0.5%	13	17+
2	0.2%	18	17+
3	0.1%	26	17+
4	0.05%	37	10+
5	0.02%	32	6+
6	0.01%	45	6+
7	0.005%	60	4+

It is apparent in the most concentrated suspensions that sea water that is more acid than Ph 7.6 destroys spermatozoa, and causes their death before they have been able to expend all their available energy (see p. 192). A post-lethal increase in acidity occurs in such suspensions. McClendon has evidently made similar observations regarding marine invertebrates, for he says: "It would be of little advantage" to determine the carbon dioxide concentrations in sea water more acid than Ph 7.6 "unless it is first absolutely established that the abnormal Ph does not make the organisms physiologically abnormal and that oxygen is still present in the water" (McClendon, Gault, and Mulholland, 1917, p. 33).

In the less concentrated suspensions which more closely simulate normal conditions it will be seen that spermatozoa that live for longer periods of time produce no more carbon dioxide than spermatozoa that live for only 4 hours. In fact the total carbon dioxide production of spermatozoa is an approximate constant. Using the carbon dioxide production as the criterion it must be concluded that the activity of spermatozoa and therefore the life of spermatozoa is limited.

An analysis of the length of life of spermatozoa is essentially, therefore, an analysis of the rate of activity of spermatozoa under varying environmental conditions.

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IV. The Effect of the Concentration of the Sperm suspension upon the Activity and upon the Longevity of Spermatozoa.

The observations and the experiments that have been cited show that sea urchin spermatozoa soon become inactive in a certain concentration of hydrogen ions or of carbon dioxide. In lower concentrations spermatozoa are the less active the higher the concentration of hydrogen ions or of carbon dioxide (see also Lillie, F. R., 1913). In the measurements of the carbon dioxide concentration of sperm suspensions that have been reported the sperm produced sufficient carbon dioxide in the more concentrated suspension to inactivate themselves in about two hours. The carbon dioxide production of inactive spermatozoa is of course very much less than the carbon dioxide production of highly active spermatozoa. Since spermatozoa are decreasingly active, the higher the concentration of carbon dioxide in the suspension, the observed falling off with time of the carbon dioxide production in all of the suspensions is easily understood.

In very concentrated sperm suspensions, therefore, the spermatozoa are active for but a short time. That undiluted mammalian sperm exhibit but little activity was observed by Koelliker. He remarks: "In der Regel findet sich die Bewegung allerdings nur am Rande des Tropfens, nicht weil hier eine Verdunstung des Samens statt hat, . . . sondern weil am Rande des Tropfens die Intercellular-flüssigkeit in etwas bemerklicherer Weise sich ansammelt" (Koelliker, A., 1856, p. 205). The observation has since been confirmed by many investigators and for nearly all forms. The explanation of the activity of spermatozoa at the border of the drop is contained in the observations of Buller (Buller, A. H., 1902) and Lillie (Lillie, F. R., 1913) quoted in the last section. At the edge of a sperm suspension the oxygen concentration is higher and the carbon dioxide concentration lower than in any other part of the drop. Consequently spermatozoa accumulate in the region of highest carbon dioxide concentration. There they are very inactive, and live for a very long time.

Further evidence that the increased length of life in the more concentrated suspensions is due to the decreased production of

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carbon dioxide in these suspensions is afforded by the following experiments, which are of two kinds. (1) The length of life of a sperm suspension was either increased by decreasing the rate at which the carbon dioxide produced by the sperm could diffuse from the suspension; or (2) the length of life of the sperm suspension was decreased by decreasing the rate at which the carbon dioxide and hydrogen ion concentration of the suspension increased. This again was attained in two ways. The procedures and the protocols of these experiments will now be reported.

V. The Relation between the Length of Life of a Concentrated Sperm Suspension and the Rate at which the Carbon Dioxide produced by Spermatozoa is Eliminated.

1. *Decreasing the Rate of Diffusion of Carbon Dioxide (Experiments 31, 32, and 33).*

In these experiments a sperm suspension was divided between vessels of different diameter. The area of the suspensions that was in contact with the air, and consequently the rate at which the carbon dioxide produced by the sperm escaped into the air constituted the only variable. It was found that the sperm suspensions from which the carbon dioxide could least rapidly diffuse lived for the longest time as judged by the percentage of eggs fertilized when spermatozoa from these vessels were added to eggs in sea water as a function of time (Table V.).

Two methods were employed of decreasing the rate at which the carbon dioxide produced by spermatozoa increases the carbon dioxide and hydrogen ion concentration of the suspension. The procedure in the one (*a*) was essentially the reciprocal of that employed in decreasing the rate of diffusion of carbon dioxide. In the other method (*b*) the "buffer" (Henderson, L. J., 1908) action of sea water and therefore the rate of neutralization of the carbon dioxide was artificially increased.

(2*a*) *Increasing the Rate of Diffusion of Carbon Dioxide.*

In experiment 214 the sperm suspension was divided between two shallow vessels. The one remained in contact with the air of the room, while over the surface of the other a stream of carbon

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Table V.

The Relation Between the Length of Life of a Concentrated Sperm Suspension and the Rate at which the Carbon Dioxide Produced by spermatozoa Diffuses from the Suspension.

Concentration of Suspension.	Time of Insemination.	Age of Sperm. Hrs.	Age of Sperm. Min.	A. Approximate Area in Square Centimeters of the Interface Between Air and Sperm Suspension. 3.	B. 20.	C. 80.	D. 500.	No. of Drops of Sperm Added to Eggs in Sea Water.
				Percentage of Eggs Fertilized when Sperm Added to Four Drops Eggs in 5 c.c. of Sea Water.				
				Experiment 32.				
0.04%	8.10	0	0	86	62	59		1
	9.30	1	20	19	10	5		
	10.10	2	00	3	1	0		
0.04%	8.10	0	0					4
	9.30	1	20	41	13	11		
	10.10	2	00	31	3	3		
	10.25	2	15	11	0	0		
				Experiment 33.				
0.05%	12.30	0	0					
	12.35	0	5	94		98	67	1
	1.20	0	50	57		43	6	
0.05%	1.20	0	50	99		94	54	8
	2.40	2	10	69		28	7	
	3.35	3	5	34		15	4	
	4.45	4	15	16		41	3	
				Experiment 31.				
0.04%	11.07	0	0	99	100			1
	12.07	1	00	100	60			
	12.37	1	30	96	21			
	12.57	1	50	99	23			
	1.30	2	23	90	14			
	2.00	2	53	61	7			
0.04%	11.07	0	0	100	100			8
	12.07	1	00	100	100			
	12.37	1	30	100	100			
	12.57	1	50	100	64			
	1.30	2	23	100	33			
	2.00	2	53	100	35			

dioxide free air was continuously drawn. In this way the carbon dioxide concentration of the one suspension was prevented from increasing at the same rate as that of the other. As a result the

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carbon dioxide production of the spermatozoa in the latter case was not so much inhibited. The life of the sperm suspension was, therefore, shortened.

TABLE VI.

THE RELATION BETWEEN THE LENGTH OF LIFE OF A CONCENTRATED SPERM SUSPENSION AND THE RATE AT WHICH THE CARBON DIOXIDE PRODUCED BY SPERMATOZOA DIFFUSES FROM THE SUSPENSION.

Experiment 214.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		A.	B.
				Approximate Carbon Dioxide Tension of the Air with which the Surface of the Suspension Was in Contact.	
				About 0.3 Mm.	About 0.1 Mm.
		Hrs.	Min.	Percentage of Eggs Fertilized when One Drop of Sperm is Added to Ten Drops Eggs in 10 c.c. of Sea Water.	
0.02%	12:00 M.	0	0		
	12.15 P.M.	0	15	100[1]	100[1]
	1.30	1	30	100[1]	100[1]
	3.30	3	30	100[1]	100[1]
	5.30	5	30	100[1]	100[1]
	7.15	7	15	100[1]	100[1]
	9.35	9	35	100[1]	85
	12.35 A.M.	13	35	100[1]	77
	1.35	13	35	100[1]	74
	10.30	22	30	90	19
	12.15 P.M.	24	15	89	20
	1.30	25	30	92	32
	5.00	29	00	93	25
	8.00	32	00	81	1
	11.50	35	50	20	0

(2b) *Increasing the Rate of Neutralization of Carbon Dioxide.*

When a mixture of a weak acid and its salt, isohydric with sea water, is added to sea water, a series of solutions is obtained that tend increasingly to maintain the reaction of the sea water. The carbon dioxide produced by spermatozoa or other organisms changes the hydrogen ion and the carbon dioxide concentration of such solutions the less, the greater the concentration of weak acids.

In the experiments to be reported mixtures of borax and boric acid were used (Palitzsch, 1914). It is, of course, necessary that neither the weak acid nor the salts of the weak acid that are

[1] Fertilization was seen to be practically complete and the percentage of eggs cleaving was only estimated.

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formed with the ions of sea water be toxic to the organism under investigation. According to a personal communication, C. M. Child has observed distinct toxic action of the concentrations of borates used in these experiments upon developing sea urchin eggs. It is therefore possible that the action of borates in shortening the life of the sperm is in part due to their toxicity.

Table VII.

The Relation between the Length of Life of a Concentrated Sperm Suspension and the Rate at Which the Carbon Dioxide Produced by Spermatozoa is Neutralized by Isohydric Borates.

Experiment 208.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		A.	B.	C.	D.	E.	F.	Ph of Borate Mixture.
				No. of c.c. of Borate Mixture Added to 10 c.c. of the Sea Water in the Sperm Suspensions.						
				0.	0.1.	0.3.	0.5.	1.0.	2.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when One Drop of Sperm Added to Five Drops Eggs in 5 c.c. of Sea Water.						
0.1%	2.25 P.M.	0	0	100	100	100	100	99	100	8.41
	2.55	0	30	100	97	98	26	47	8	
	3.25	1	00	92	82	57	55	10	1	
	4.00	1	35	52	21	14	13	0	0	

Experiment 205.

0.2%	11.20 A.M.	0	0	100	100	100	100	100		8.41
	11.50	0	30	100	100	100	99	100		
	1.40 P.M.	2	20	66	28	40	4	2		

Experiment 209.

1.0%	10.00 A.M.	0	0	100					50	8.41
	5.20 P.M.	7	20	98	25	6	1	3	0	

Experiment 223.

1.0%	4.00 P.M.	0	0							8.08
	10.00 A.M.	18	0	88	80	77	70			
	11.30 A.M.	19	30	85	86	27	87			
	[1] 2.00 P.M.	22	00	100	100	28	19			
1.0%	4.00 P.M.	0	0							8.51
	10.00 A.M.	18	0	88	82	3	8			
	11.30	19	30	85	80	2	2			
	2.00 P.M.	22	00	100	100	9	27			

[1] The eggs of a female that had been opened at 1.30 P.M. were used in this series of inseminations. They were evidently more easily fertilized than the eggs of the female that had been opened at 9.30 A.M.

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In suspensions with the highest concentration of weak acids the spermatozoa lived for the shortest time. For the higher the concentration of weak acids the smaller the change in the hydrogen ion and carbon dioxide concentration of the suspensions due to the carbon dioxide produced by the spermatozoa. Consequently spermatozoa in more alkaline solutions, where they were most active, lived for the shortest time. In order the better to prevent spermatozoa from being inactive, a borate mixture that was slightly more alkaline than sea water was employed in the first three experiments reported (Experiments 205, 208, 209). The hydrogen potential of the borate mixture is recorded in the last column of Table VII.

That even this slight change in the hydrogen potential of the borate effects spermatozoa is demonstrated in Experiment 223 where slightly different mixtures of borates were used in the same concentration. It will be seen that spermatozoa in the suspension to which had been added the more alkaline mixture, lived the shorter time.

VI. The Effect of the Hydrogen Ion Concentration of the Suspension upon the Activity and upon the Longevity of Spermatozoa.

The activity of spermatozoa is a function of the hydrogen ion concentration. Repeated observation has confirmed this relation. Since there is a definite relation between the activity and the length of life of spermatozoa, the latter is also a function of the hydrogen ion concentration. The hydrogen ion concentration of the ocean at Woods Hole, Massachusetts, is about 0.1×10^{-7} (Ph 7.95 to Ph 8.15). The weak acids (Henderson, L. J., and Cohn, E. J., 1916) and the currents prevent the hydrogen ion concentration of the ocean from appreciably changing. In such a solution the length of life of spermatozoa is short.

Loeb has observed the simultaneous spawning of the sea urchins (*Strongylocentrotus purpuratus*) at the shore of Pacific Grove. "At such spawning seasons the sea water becomes a suspension of sperm" (Loeb, J., 1916, p. 94). It would be interesting to know whether the hydrogen ion concentration of such a suspension increases.

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Such concentrations of spermatozoa as were used in the experiments reported in the last section probably never occur in the ocean. The conditions that obtained in these experiments approximate those of ripe sperm in the testes very much more closely than they do those of sperm that are shed into the ocean. The hydrogen ion concentration in these suspensions increased not inconsiderably as a result of carbon dioxide produced by the sperm. This rise in carbon dioxide (and also in hydrogen ion) concentration was measured. The hydrogen ion and carbon dioxide concentration of the suspensions in which spermatozoa lived for the longest time was sufficiently great to inactivate spermatozoa. The length of life of concentrated sperm suspensions is therefore for the most part ascribable to the hydrogen ion concentration of such suspensions.

The length of life of a sperm suspension at different hydrogen ion concentrations was determined.

Two criteria of the length of life of the sperm were employed. The fertilization tests are reported. The hydrogen potential of the sea water was determined by colorimetric comparison with standardized mixtures of borates or phosphates in the way that that has already been described (p. 171).

In the experiments to be reported the hydrogen ion concentration was decreased by the addition of sodium hydroxide to sea water. The hydrogen ion concentration was increased by the addition of hydrochloric acid.

When an acid stronger than carbonic acid is added to sea water, carbonic acid is displaced from its salts and carbon dioxide is set free. As the carbon dioxide that is set free diffuses from the solution the hydrogen ion concentration decreases, until the carbon dioxide of the sea water is again in equilibrium with the partial pressure of that gas in the air. The hydrogen ion concentration that is eventually reached is different from the original hydrogen ion concentration of the sea water, but nearer to it than to the hydrogen ion concentration immediately after the acid is added. This regulation of the neutrality persists until all of the carbonates have been decomposed. The rate at which equilibrium is approached depends upon the temperature, the surface, and the degree of agitation.

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Because of this property of carbonate solutions it is necessary to know not only the hydrogen ion concentration but also the carbon dioxide tension. The latter is, as stated, above expressed by the number of millimeters of mercury that represents the partial pressure of the gas. The procedure usually followed was to restore the equilbrium between the sea water (of whatever hydrogen ion concentration) and the carbon dioxide of the air, before beginning an experiment. This was accomplished either by shaking with air, or by bubbling air through the solutions.

In a series of experiments it was found that the more alkaline the solution (*i. e.*, the lower the hydrogen ion concentration) the shorter the life of the sperm. The activity of the spermatozoa is increased in these suspensions, and spermatozoa that are added to ripe eggs in sea water while in this activated condition have a greater "fertilizing power" (this has been previously reported. See Fuchs, H. M., 1915) than spermatozoa that have been in less alkaline sea water. This lasts for a much shorter time, since the life of spermatozoa is very short in alkaline solution. (If the hydrogen potential is greater than about Ph 9.4 spermatozoa are instantly agglutinated.) In order to demonstrate the increased "fertilizing power" of spermatozoa that have been in alkaline solution it is necessary to inseminate in such dilution that the spermatozoa that have been in sea water with greater hydrogen ion concentration do not fertilize all of the eggs.

Experiment 227 illustrates both the effect of alkaline sea water in increasing the "fertilizing power" for a short time and of more acid sea water in increasing the time during which the "fertilizing power" is exhibited. The reversal in the effect of alkaline sea water upon the "fertilizing power" of spermatozoa was demonstrable only because the eggs used in the first part of this experiment were fertilized with difficulty. Otherwise the early fertilizations would have been complete, and the effect of alkaline sea water upon spermatozoa not have been observed. Diagram III. represents the prolongation of the life of the sperm in acid suspension. The ordinates measure the fertilizing power of the sperm at the times designated by the abscissæ. The hydrogen potentials of the suspensions are symbolically recorded.

If sea water is much more alkaline than Ph 9.4, spermatozoa

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are instantly agglutinated. If sea water is about Ph 7.6, spermatozoa are inactive. Sea water that is much more acid than Ph 7.6 not only tends to inactivate, but also to destroy spermatozoa and the more so the higher the hydrogen ion concentration and the longer the sperm are subjected to these acidities.

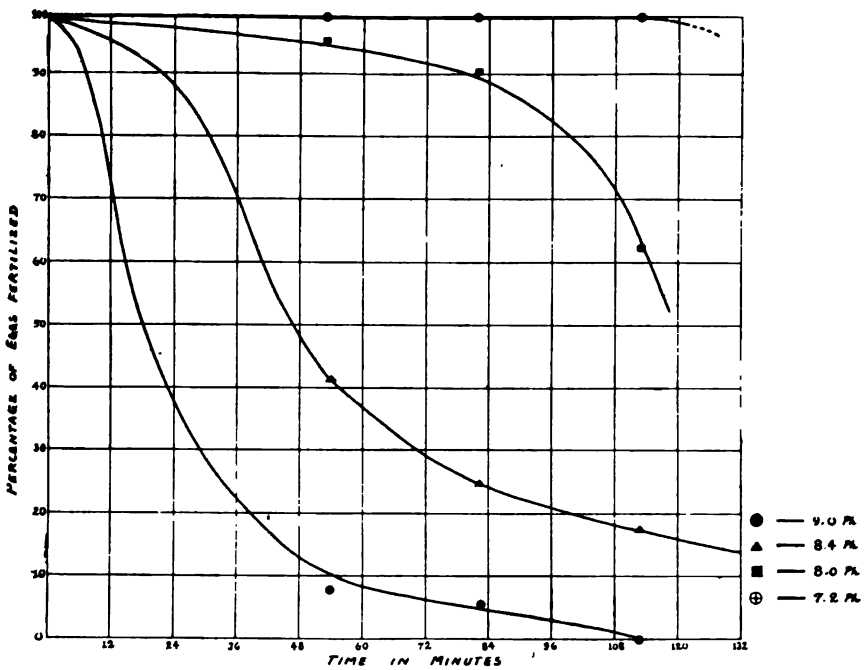

DIAGRAM III.

In sea water that is less acid than Ph 7.6 spermatozoa live the longer the higher the hydrogen ion concentration. The more inactive the spermatozoa the more slowly their "fertilizing power" diminishes when they are transferred to sea water where they are reactivated.

VII. The Relation between the Activity and the "Fertilizing Power" of Spermatozoa.

"Within a wide limit of egg-concentration the important factors in fertilizing power of sperm suspensions are: (1) concentration, (2) time" (Lillie, F. R., 1915, p. 246). The results of this investigation confirm this general conclusion of Lillie's, and

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add one more factor, the hydrogen ion concentration. For the length of life and the "fertilizing power" of a sperm suspension are dependent on the hydrogen ion concentration of the suspension, and the "fertilizing power" of spermatozoa at the same hydrogen ion concentration is in some way dependent upon the sperm concentration.

The Effect of the Hydrogen Ion Concentration upon the Fertilizing Power of Spermatozoa.

The loss of "fertilizing power" of active sperm suspensions of approximately the same concentration (0.04 per cent.) at different hydrogen ion concentrations is graphically represented

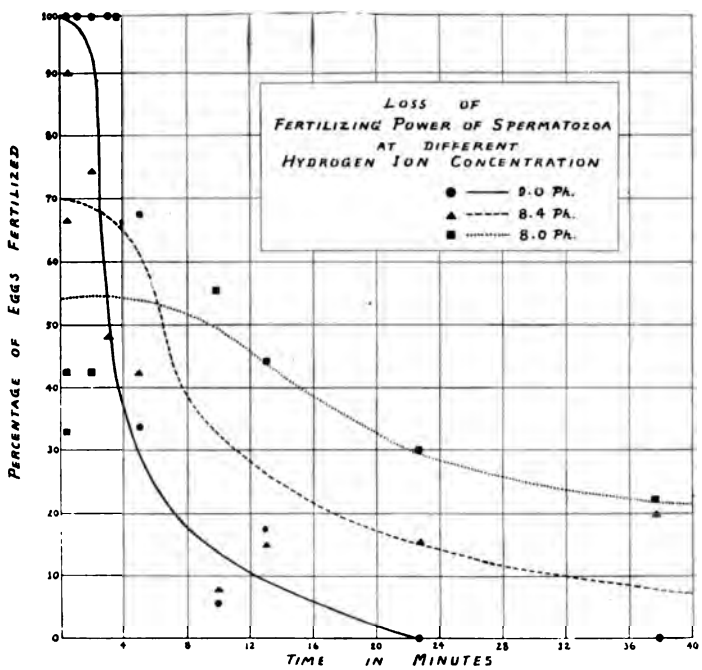

Diagram IV.

in Diagram IV. The curves are plotted from the data in Table VIII. on the basis of maximum "fertilizing power" at Ph 9.0. If but 50 per cent. of the eggs were fertilized at Ph 9.0 (as in experiment 227–I.) and 24 per cent. at Ph 8.4 the ratio of the "fertilizing powers" is of course as 100 to 48.

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TABLE VIII.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION OF THE SUSPENSION UPON THE FERTILIZING POWER AND UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		C.c. of 0.02N HCl to 10 c.c. Sea Water.			C.c. of 0.2N NaOH to 10 c.c. Sea Water.					No. Drops of Sperm Added to Eggs.
				1.1.	1.0.	0.5.	0.	.06.	.12.	.25.	.50.	
				Hydrogen Potential of Sperm Suspensions at CO_2 Tension of Air.								
						7.7.	8.0.	8.2.	8.4.	8.6.	9.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm is Added to Five Drops Eggs in 5 c.c. of Sea Water.								
Experiment 20.												
0.015%	11.51		0									1
	11.53		2				41	48	67	51	98	
	1.00	1	9				0	0	0	0	0	
Experiment 226.												
0.04%	4.40		0									1
	4.42		2				20	39	35	47	47	
	4.45		5				32	18	20	18	16	
	4.50		10				26	32	4	34	3	
Experiment 21.												
0.045%	12.25		0				33	94	90	90	99	4
	1.25	1	0				5	36	23	1	3	
	2.20	1	55				1	7	7	0	1	
Experiment 227—I.												
0.08%	11.07		0									1
	11.10		3	4	38	7	51		24		50[1]	
	11.20		13	11	60	15	22		8		9	
	11.30		23	19	31	18	15		8		0	
	11.45		38	13	44	0	11		10		0	
Experiment 227—II.[2]												
	12.00		53	100	100	89	96		41		8	
	12.30	1	23	100	100	67	90		25		6	
	1.00	1	53	100	100	60	63		17		0	
Experiment 22.												
0.7%	12.25		0									1
	12.40		15				100	100	100	100	100	
	1.15		50				98	100	100	100	100	
	5.20	4	55				35	86	90	5	0	

[1] But 0.3 c.c. of sodium hydroxide had been added in Expt. 227. The Ph was 8.8.

[2] Same sperm used as in 227—I. The eggs, however, of another female.

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Spermatozoa that have been in alkaline sea water have a greater "fertilizing power" than spermatozoa that have been in sea water at the hydrogen ion concentration of the ocean. This becomes apparent only at dilutions such that the spermatozoa that have been in the less alkaline sea water do not fertilize all of the eggs. If all of the eggs are not fertilized, it must be either (*a*) because there are not enough spermatozoa or (*b*) because the spermatozoa are not sufficiently potent. Since the same concentration of sperm is able to fertilize completely at another hydrogen ion concentration, the first alternative does not explain the results in these experiments (Experiment 20, 21, 226, 227–I.).

If the "fertilizing power" of active spermatozoa were exactly proportional to the energy expended, the areas beneath the curves in the diagram would be exactly equal, since the total available energy of spermatozoa is practically constant (p. 183). If the relation is not as simple as this, it is at all events perfectly definite and definable, and strongly suggestive of a simple functional relation, at least during the first few minutes of the life of the sperm.[1]

The Effect of the Sperm Concentration upon the "Fertilizing Power" of Spermatozoa.

"In his epoch-making 'Expériences pour servir a l'histoire de la génération des animaux et des plantes' published in 1785 the Abbe Spallanzani describes among his numerous experiments on fertilization and artificial parthenogenesis some determinations concerning the minimal quantity of sperm necessary to fertilize the eggs of the frog" (Lillie, F. R., 1915, p. 229). "In 1824 Prevost et Dumas confirmed these" results (Lillie, F. R., 1915, p. 229). More recently in the "Analysis of Variations in the Fertilizing Power of Sperm Suspensions of *Arbacia*" (Lillie, F. R., 1915, p. 229) that has already been quoted, F. R. Lillie demonstrated that at "a dilution of 1/10,000 per cent.," where "one can rarely find a single spermatozoön in the jelly of the fertilized

[1] Thereafter it is difficult to conceive of the physiological condition of the spermatozoön as suffering no alteration. A change in the physiological condition must in turn affect the "fertilizing power." Strong evidence for the view that the ageing of Echinid sperm affects its physiological condition has previously been presented by Dungay (1913) and Vernon (1899).

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TABLE IX.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION OF THE SUSPENSION UPON THE FERTILIZING POWER AND UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 228.

Concentration of Suspension.	Time of Insemination.	Age of Sperm. Hrs.	Age of Sperm. Min.	C.c. of .02N HCl to 10 c.c. Sea Water. 1.0.	0.9.	0.8.	0.5.	0.0.	No Drops of Sperm Added to Eggs.
				Hydrogen Potential at CO_2 Tension of Air.		7.4.	7.7.	8.0.	
				Percentage of Eggs Fertilized when Sperm is Added to Five Drops Eggs in 5 c.c. of Sea Water.					
0.001%	11.55		0						1
	12.00		5		6	12	31	19	
	12.15		20		1	0	4	2	
	1.00		65		0	0	0	0	
0.005%	11.55		0						1
	12.00		5		41	71	72	86	
	12.15		20		1	0	3	29	
	1.00		65		0	0	1	0	
0.01%	11.55		0						1
	12.00		5		71?	31	94	100	
	12.15		20		7	1	78	86	
	1.00		65		0	0	33	5	
Experiment 215.									
0.25%	12.05		0						1
	12.30		25	84		78	86	84	
	9.00	8	55	12		66	87	6	
	12.40	12	35	0		7	23	0	

eggs" (Lillie, F. R., 1915, p. 234) about 95 per cent. of the eggs were fertilized if the time, which "is an extremely important factor with reference to fertilizing power" (Lillie, F. R., 1915, p. 234) during which the sperm were in sea water, was short. For: "If the sperm suspensions lose their fertilizing power with time, it must be that the significance of time in this respect varies inversely to concentration" (Lillie, F. R., 1915, p. 239).

I believe that Lillie's results have conclusively demonstrated that one sperm is quite sufficient to fertilize an egg if it has not already expended a large part of its available energy. Thereafter the statement that "the initiation of development by a single spermatozoön . . . is impossible because a single sperm

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cannot affect those changes in the egg-coverings" (Glaser, O., 1915, p. 153) which are necessary to fertilization, has unquestioned significance. The older the spermatozoa and the more their available energy has been expended, the more spermatozoa are necessary in order to effect fertilization. There is, however, even then no doubt that "a single spermatozoön is sufficient to carry out the bi-parental effect" (Glaser, O., 1915, p. 153).

The "fertilizing power" of sperm at the same hydrogen ion concentration is, therefore, in some way dependent on the concentration of the sperm. In Experiment 228 in Table IX. (p. 196) a 0.001 per cent.; a 0.005 per cent. and a 0.01 per cent. suspension was made from the sperm of one male. The "fertilizing power" at each hydrogen ion concentration was lost the sooner the smaller the concentration of sperm.

It is, of course, true that in all but the most dilute suspensions the hydrogen ion concentration will in a short space of time be the higher the more concentrated the suspension as a result of the carbon dioxide produced by the sperm. This probably occurred in Experiment 22, but it is improbable that this was the only factor in the other experiments, and it certainly was not a factor in the data that will now be presented.

In Experiments 31, 32 and 33 (p. 186) different amounts of sperm from the same suspension were added to the same concentration of eggs. These experiments are retabulated in order that the percentage of eggs that were fertilized by different amounts of sperm from the same suspension at the same time can more easily be compared. When all of the eggs were not fertilized by both concentrations of sperm the number of eggs fertilized was always the greater the more spermatozoa were added.[1]

This is not a new observation. It is the common experience of investigators, and may be explained either by assuming that in the greater concentration more sperm will on the average arrive at the periphery of the egg with sufficient action (having the dimensions of energy $\times$ time) to initiate development, or by assuming that mass action of spermatozoa may be effective in

[1] It should be pointed out that whereas motility is unquestionably a manifestation of energy, the observation of motile spermatozoa at the periphery of an unfertilized egg in no way indicates the physiological condition of the spermatozoa.

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initiating the development of the egg. This implies that what is true in parthogenesis is also true in normal fertilization. R. S. Lillie (Lillie, R. S., 1916) has quite beautifully shown that the product of the concentration of the parthenogenetic agent and the length of time during which it is necessary to subject eggs to the agent in order to initiate development is a constant. The evidence that has been presented strongly suggests a similar quantitative relation in fertilization.

TABLE X.

THE RELATION BETWEEN THE CONCENTRATION OF THE SPERM AND THE PERCENTAGE OF EGGS THAT ARE FERTILIZED.

Number of Experiment.	Designation of Suspension.	Age of Sperm Suspension.		Amount of Sperm Added.		Amount of Sperm Added.	
		Hrs.	Min.	1 Drop.	4 Drops.	1 Drop.	8 Drops.
				Percentage of Eggs that are Fertilized.		Percentage of Eggs that are Fertilized.	
32	*A*	1	20	19	41		
	B	1	20	10	13		
	C	1	20	5	11		
	A	2	0	3	31		
	B	2	0	1	3		
	C	2	0	0	3		
33	*A*		50			57	99
	B		50			43	94
	D		50			6	54
31	*A*	1	0			100	100
	B	1	0			60	100
	A	1	30			96	100
	B	1	30			21	100
	A	1	50			99	100
	B	1	50			23	64
	A	2	23			90	100
	B	2	23			14	33
	A	2	53			61	100
	B	2	53			7	35

VIII. THE EFFECT OF THE CARBON DIOXIDE CONCENTRATION UPON THE ACTIVITY AND UPON THE LONGEVITY OF SPERMATOZOA.

The effect of increasing the carbon dioxide concentration of the suspension is to increase the length of life of the spermatozoa.

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TABLE XI.

THE EFFECT OF THE CARBON DIOXIDE TENSION UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 225.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		CO_2 Tensions of the Suspensions in Mm.					No. Drops Sperm Added to Eggs.
				16.	4.	2.	1.	0.3+.	
				Hydrogen Potentials of the Suspensions.					
				6.5.	7.0.	7.3.	7.6.	8.0.	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm Added to Eggs in 5 C.C. of Sea Water.					
0.04%	4.40	0	0						1
	9.00	4	20	10	7		97	53	
	11.45	7	5	0	4		41	16	
Experiment 212.									
0.2%	12.05	0	0						1
	12.10		5	100	100		100	100	
	12.25		20	100	100		100	100	
	2.30	2	25	100	100		100	100	
	4.40	4	35	99	100		100	100	
	11.50	11	45	100	100		100	100	
	9.55	21	50	7	4		94	80	
	12.20	24	15	1	0		100	100	
	4.50	28	45	2	15		92	90	
Experiment 213.									
0.2%	12.10	0	0						1
	12.20	0	10			100		100	
	1.50	1	40			100		55	
	3.30	3	20			98		96	
	4.50	4	40			100		99	
	9.40	9	30			99		95	
	9.40	21	30			100		20	
	12.15	24	5			84		5	

For as the carbon dioxide concentration of sea water increases, so also does the concentration of carbonic acid and of hydrogen ions. In several experiments the length of life of a sperm suspension at different carbon dioxide tensions[2] was determined and sperm were found to live for the longest time when the carbon dioxide tension was about one millimeter. The hydrogen potential of sea water at a carbon dioxide tension of one milli-

[1] In this experiment five drops of eggs were added to the undiluted suspensions.

[2] The relation between the tension and the concentration of carbon dioxide is discussed on page 172.

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meter is about 7.6 (Henderson, L. J., and Cohn, E. J., 1916). But this is the hydrogen potential that has been found to be most effective in increasing the length of life of spermatozoa. Three experiments are reported in Table XI. It will be seen that when the carbon dioxide tension is greater than one millimeter and the hydrogen potential less than 7.6, sperm are destroyed. This is in complete agreement with what has already been found with respect to the hydrogen ion concentration.

It is not maintained that the only effect of carbon dioxide upon the physiological condition of spermatozoa is brought about by ionized hydrogen. On the contrary, several experiments, which are unfortunately not conclusive (and are therefore not reported) indicate a difference in the subsequent behavior of spermatozoa that are subjected to the same hydrogen ion concentration but to different carbon dioxide tensions. The fact that the total carbon dioxide production per unit concentration of sperm in concentrated suspensions is less than in more dilute suspensions (Table IV.) suggests that the rate at which carbon dioxide is eliminated depends in some way upon the difference in carbon dioxide tension between the spermatozoon and its environment.

IX. The Effect of the Oxygen Concentration upon the Activity and upon the Longevity of Spermatozoa.

The generalization may be hazarded that whatever decreases the activity increases the length of life of spermatozoa and conversely that whatever increases the activity decreases the length of their life. Buller (Buller, A. H., 1902) observed the differential activity of spermatozoa in an oxygen gradient (see p. 175). Drzewina and Bohn (Drzewina, A., and Bohn, G., 1912) have demonstrated that sperm live for a long time in an oxygen-poor medium.

Drzewina and Bohn have also demonstrated that the addition of KCN to sea water prolongs the life of the sperm. Loeb (1915) has shown that spermatozoa are immobilized by NaCN, and it is certain that the length of their life is thereby increased. "It is a well-known fact that the unfertilized eggs of the sea urchin (in fact of all marine animals) perish when they lie for some time in

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sea water and one of the main causes of this phenomenon is also known, namely oxidations. If the oxidations are inhibited through the removal of oxygen or the addition of KCN the life of the eggs can be prolonged"[1] (Loeb, J., 1915, p. 282).

It is probable that oxygen lack also plays a part in increasing the length of life of concentrated sperm suspensions. McClendon (McClendon, Gault and Mulholland, 1917) has recently estimated that respiration that raises the hydrogen potential of sea water (of excess base 23) to approximately 7.6 uses up all of the available oxygen.

The experiments of Drzewina and Bohn have been repeated and the results substantially confirmed. Spermatozoa are quite inactive in the concentrations of KCN that are most effective in prolonging the life of spermatozoa. The results of several experiments follow.

TABLE XII.

THE EFFECT OF THE ADDITION OF KCN TO SEA WATER ON THE LENGTH OF LIFE OF THE SPERM SUSPENSION.

Experiment 14.

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		Number of C.C. of 0.1*N* KCN Added to a Liter of Sea Water.								No. of Drops Sperm Added to Eggs.
				10	2.5	1.25	0.62	0.31	0.16	0.04	0	
		Hrs.	Min.	Percentage of Eggs Fertilized when Sperm Added to Eggs in 5 C.C. Sea Water.								
0.04%	11.05	0	0	99	97		97		93	97	71	8
	12.05	1	0	100	98						91	
	1.40	2	35	1	100						34	
	2.45	3	40	2	100		79		16	26	4	
	7.15	8	10		73							

Experiment 27.

0.7%	3.45	0	0									1[2]
	4.40	0	55		100	100	100	100			100	
	9.50	18	5		100	100	100	100			100	
	9.45	42	0		100	5	99	98			97	
	2.00	46	15		98	0	4	2			2	
	4.45	49	0		97		6	0			0	
	7.00	51	15		2		1					

[1] The way in which KCN effects the oxidations of cells need not be discussed in connection with these experiments. For a discussion of this question see Lillie, R. S., 1916, page 311, and Child, C. M., 1915, p. 66).

[2] Insemination took place in 10 c.c. of sea water in this experiment.

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X. The Effect of Beef Broth upon the Activity and upon the Longevity of Spermatozoa.

Gemmill showed that the length of life of spermatozoa could be increased by adding beef broth to sea water. He believed that this brought about the "keeping alive of spermatozoa by artificial nutrition" (Gemmill, J. F., 1900, p. 171) (see p. 178).

The work of Gemmill has been repeated and his observation that sea water to which beef broth has been added in appropriate concentration prolongs the life of spermatozoa, confirmed. The activity of spermatozoa, however, is decreased in the beef broth suspensions. Moreover the addition of beef broth to sea water increases the hydrogen ion concentration.[1]

It is unfortunately not possible to neutralize the suspension without throwing down a heavy precipitate.[2] This brings about such changes in the ionic composition of the sea water as to make uninterpretable the results of experiments carried out in such a medium.

The protocols of two experiments that confirm the results of Gemmill are recorded.

2.5 grams of Armour's "extract of beef" were dissolved in 290 c.c. of sea water, making the concentration by weight approximately 8.6 per cent. The suspension of beef broth in sea water was acid to neutral red. That is, the hydrogen ion concentration was greater than Ph 6.5. Diluting the suspension at once decreased its concentration in beef broth and in hydrogen ions. The hydrogen ion concentrations were not directly determined, but their approximate value has been calculated from the number of cubic centimeters of 0.01 N NaOH required to make them alkaline to phenolphthalein.

To 10 c.c. of the different concentrations of acid broth one drop of dry sperm was added. Tests were made by adding one drop of each sperm suspension to five drops of eggs in 10 c.c. of sea water. The percentage of eggs that were fertilized are reported in Table XIII.

[1] The increase in viscosity of the suspension, and also the increase in protein content, may be effective in decreasing the activity of spermatozoa.

[2] In all probability an insoluble calcium salt or aggregate.

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TABLE XIII.

THE EFFECT OF THE ADDITION OF BEEF BROTH TO SEA WATER UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Experiment 35.

Concentration of Suspension.	Time of Insemination.	Age of Sperm. Hrs.	Age of Sperm. Min.	Concentration of Beef Broth in Sea Water. 8.6%.	4.3%.	2.1%.	1.1%.	0.5%.	0.0%.	No. of Drops Sperm Added to Eggs.
				Probable Hydrogen Potential of the Suspensions. 6.5.	7.2.	7.6.	7.8.	7.9.	8.0.	
				Percentage of Eggs Fertilized when Sperm Added to Five Drops Eggs in 10 C.C. of Sea Water.						
0.02%	11.55	0	0	61	81	99	99		93	1
	12.25	0	30	2	92	100	58		0	
	12.48	0	53	0	61	88	99		2	
	1.15	1	20	0	5	79	67		0	
	1.45	1	50	0	0	2	46		0	
Experiment 34.										
0.7%	10.05	18	35	0	98	99	98	99	97	1
	2.45	23	15	0	94	97	95	98	96	
	6.20	26	50	0	53	95	94	79	30	
	10.10	30	40	0	32	89	98	43	20	

XI. THE EFFECT OF "EGG WATER" UPON THE ACTIVITY AND UPON THE LONGEVITY OF SPERMATOZOA.

Owing to the fact that sperm are "agglutinated" by the water in which eggs of the same or closely related species have been allowed to stand, much interest has centered around the effects of the so called "egg water" or "fertilizin" upon the fertilizing power of spermatozoa.

A. H. Buller seems to have been one of the first students of the fertilization process in Echinids to have noticed this phenomenon. In 1900 he reported before the British Association: "In the case of *Arbacia* it was discovered that when spermatozoa are introduced into a drop containing freshly extruded eggs they collect into small balls, often composed of 100 or more individuals. The balls were also formed after the water had received four successive filtrations. A tactile stimulus appears to play a part in the phenomenon" (Buller, A. H., 1900, p. 387).

Since then this phenomenon and the properties of the agglu-

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tinating "egg water"[1] have been successively studied by E. von Dungern (1901) and (1902); A. Schücking (1903) J. De Meyer (1911); F. R. Lillie (1912, 1913, 1914, 1915); H. M. Fuchs (1915); Jacques Loeb (1914, 1915); Otto Glaser (1913, 1914); A. Richards and A. E. Woodward (1915) and A. E. Woodward (1915). Each investigator has conceived the function of the "egg water" and its importance in the fertilization process to be different. It is not the purpose of the present communication to consider the function of the "egg water" (although that is a problem of great biological interest) but the behavior of the spermatozoön, and it has been possible to repeat and to explain many of the seemingly contradictory observations of different investigators on the effect of "egg water" upon the fertilizing power and upon the length of life of spermatozoa.

The effect of "egg water" upon spermatozoa as was clearly shown in the admirable investigation of Schücking depends upon the relative concentration of egg water and sperm; upon the absolute concentration of each; and upon the length of time during which sperm are allowed to remain in the egg water. Schücking observed that: "Die sauer reagirende Eimasse übt bei den genannten Echinodermen eine tödtliche, bei kurzer Dauer der Einwirkung lähmende, in geringer Menge agglutinirende bezw. erregende und anlockende Wirkung auf Spermien der eigenen und fremden Art aus" (Schücking, A., 1903, p. 91).

In a more complete analysis of the phenomenon of activation and agglutination F. R. Lillie (1913) showed that if "egg water" is added to a sperm suspension the activity of the spermatozoa is greatly increased. One of the manifestations of this increased activity is the "agglutination" phenomenon. According to Gray "if a drop or two of a very weak solution of cerous chloride is added to a suspension of *Arbacia* sperm in sea water the spermatozoa become intensely active, and rapidly aggregate into clumps" (Gray, J., 1915, p. 123). This may possibly be (Lillie, F. R., 1915, p. 20) what Lillie now calls "mass coagulation," which was described by Loeb in 1904 (Loeb, J., 1904) and is favored not only by increase in the hydroxyl ion concentration

[1] The distilled water "extract" of Echinid eggs has been found to possess many of the properties of the "egg water."

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but by increase in the concentration of bivalent ions, notably calcium (Loeb, J., 1914). Unlike "agglutination" it is not reversible. In an earlier paper Lillie did not distinguish between the two phenomenon, for he wrote: "Agglutination is not in itself a specific process; it may take place spontaneously to a certain extent under some conditions; it is caused by increase of alkalinity of the sea-water" (Lillie, F. R., 1913, p. 563). Loeb has designated Lillie's "agglutination" as "cluster formation."

It will be remembered that increasing the alkalinity also increases the activity of spermatozoa. Loeb (1914) has shown that inactive sperm do not exhibit the reversible "agglutination."[1] The irreversible agglutination, or "mass coagulation" is independent of the motility of the spermatozoa.

If the "egg water" is of sufficient strength,[2] however, the sperm are completely non-motile after the initial period of activation. By adding eggs to such spermatozoa Lillie showed that their fertilizing power was slight. "The powerful effect of the egg extract on spermatozoa of the same species may be shown by a complete loss of motility as we have already seen, and also by a corresponding loss or diminution of the fertilizing power" (Lillie, F. R., 1913, p. 558).

Fuchs (Fuchs, H. M., 1915) in experiments in which sperm that had been treated with "egg water" were added to eggs in sea-water showed that the fertilizing power of the sperm had been increased by the "egg water."[3]

But an analysis of the effect of "egg water" upon the fertilizing power of spermatozoa must differentiate between the following

[1] That "agglutination" is reversible may possibly be attributable to an increase in the acidity of the clusters; the result of the carbon dioxide produced by the tremendously active spermatozoa. In alkaline medium the carbon dioxide would be neutralized.

[2] In the measurement of the strength of "egg water" the method of F. R. Lillie, (namely considering that dilution of "egg water" that gives a visible "agglutination" as unity), is adopted. Reference is made to the papers of Lillie, F. R., and Fuchs, H. M., for a detailed description of the methods and precautions employed in this type of experimentation.

[3] According to T. B. Robertson, "when spermatozoa are washed in 3/8 *M* $SrCl_2$ and then in blood serum, they gain an added potency in fertilizing." (Robertson, T. B., 1913, p. 128.) The same treatment agglutinates (*ibid.*, page 71) (and also cytolyzes) (*ibid.*, p. 91) the eggs of the sea urchin.

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effects of "egg water" upon the physiological condition of spermatozoa.

When sperm are added to egg water, their activity is tremendously increased. If they are then immediately transferred to the ripe eggs of the same species in sea water it is to be supposed that their fertilizing power will for a very short time be at least as great, if not greater than the fertilizing power of less active sperm. The experiments by Fuchs seem to have been conducted in this way. The experiments which are recorded in Table XIV. were carried out in such a way as to make the time during which the sperm were in the "egg water" as short, and the volume of sea water in which the sperm were added to eggs as great, as possible. A few experiments appear to agree with those of Fuchs in that the "egg water" increased the fertilizing power of the sperm. The results are irregular, however, for if the concentration of the "egg water" is too great, or if the time during which spermatozoa are in the "egg water" is too long, so that the activity of the spermatozoa is decreased, the fertilizing power of spermatozoa is not so great as is that of spermatozoa that have been in sea water. This seems to have been the case in the experiment of Lillie quoted by Fuchs; in which "to five watch glasses containing each eight drops of water or of different concentrations of egg-extract were added three drops of 'opalescent' sperm-suspension. After 12 minutes, a drop of a suspension of fresh eggs was added to each.' 5 per cent. of the eggs in the water segmented, but none of those in the four different concentrations of extract" (Fuchs, H. M., 1915, p. 275; Lillie, F. R., 1913, p. 558). It will be noted that in this experiment of Lillie's insemination took place in the egg extracts. Repeating this procedure as nearly as possible, Fuchs was able to obtain higher percentages of fertilization in his "extracts" than in the sea water. This difference in the effect of egg "extracts" upon the fertilizing power of spermatozoa is probably due to the relative concentration of the "extract" and the sperm, and to the absolute strength of the former. For, as Schücking early observed, although spermatozoa are stimulated by low concentrations they are "lamed" (that is, their activity is temporarily decreased) by

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TABLE XIV.

THE EFFECT OF "EGG WATER" UPON THE FERTILIZING POWER OF SPERMATOZOA.

Concentration of Suspension.	Concentration at Insemination.		Age of Sperm.		Approximate Concentration of "Egg Water."							
	Drops of Sperm.	Vol. of Sea Water.	Hrs.	Min.	640.	128.	64.	16.	8.	4.	1.	0.
					Percentage of Eggs Fertilized by Sperm.							
Experiment 8.[1]												
0.04%	1	50 c.c.	0	0				44		59		12
			0	5				17		9		14
	1	200 c.c.	0	0				18		52		23
			0	5				36		11		9
Experiment 12.												
0.04%	1	50 c.c.	0	2			98		87		86	78
			0	5			41		76		58	37
			0	10			75		34		10	77
	8	50 c.c.	3	10			22		18		25	14
Experiment 11.[2]												
0.04%	1	50 c.c.	0	0		19				11		16
			0	5		15				21		10
			0	10		11				38		
Experiment 16.												
0.013%	1	5 c.c.	0	3	48		53	52		42	45	27
											81	
			1	22	43		62	10		8	6	11
Experiment 5.[3]												
0.04%	8	5 c.c.	0	5				49		36	28	19
			0	10				21		26	6	2
			1	00				37		27	8	4
			2	00				42		8	3	2
			5	15				0		0	0	0
Experiment 10.												
0.04%	1	50 c.c.	0	3			68	75		95	94	100
			0	8			89	10		83	21	94
			3	50			87	10		61	67	62

[1] In Experiment 8 the strength of the "egg waters" was as 3 to 1, but the "egg water" gave a 60-second reaction.

[2] In Experiment 11 the strength of the "egg waters" was as 125 to 5.

[3] In Experiment 5 the strength of the "egg waters" was as 16:4:1.

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higher concentrations of egg "extract."[1] In certain of the experiments that are recorded the "fertilizing power" of the sperm was increased in certain concentration of "egg water" but decreased in greater concentration.

TABLE XV.

THE EFFECT OF "EGG-WATER" UPON THE LENGTH OF LIFE OF SPERMATOZOA.

Concentration of Suspension.	Concentration at Insemination.		Age of Sperm.		Approximate Concentration of "Egg Water."									
					1280.	640.	128.	64.	32.	16.	8.	?.	2.	0.
	Drops of Sperm.	Vol. of Sea Water.	Hrs.	Min.	Percentage of Eggs Fertilized by Sperm.									
Experiment 17.[2]														
0.013%	2	5 c.c.	1	0		38	57	76						7
Experiment 13.[3]														
0.04%	8	5 c.c.	0	2	100		100			100			100	100
			1	0	100		100			99			96	91
			2	2	98		91			64			42	60
			3	40	99		82			60			26	30
			5	0	95		40			7			8	10
			7	0	54		8			1			2	2
Experiment 19.[4]														
1.0%	1	6.7 c.c.	19	40		66	97			99				0
			24	35		16	77			100				0
			43	40		0	0			0				0

Where the relative concentration of the "egg water" and sperm is such that the activity of the sperm is decreased, the length of life of the sperm, as measured by their ability subsequently to fertilize the ripe eggs of the same species in sea water, is greater than is that of sperm that exhibit constant activity in sea water for an equal length of time. This is to be expected since the activity of spermatozoa is limited (Cohn, E. J., 1917). Whereas the fertilizing power of sperm that have been in "egg water" of various concentrations for but a few moments is often

[1] All of the properties of "egg water" and "egg extract" are not the same. Sperm are, however, activated and agglutinated by both.

[2] The actual strength of the "egg water" used in Experiment 17 is not known. In all three suspensions, however, it gave at least a three minute agglutination.

[3] An egg extract was used in Experiment 13.

[4] An egg extract was used in Experiment 19. The strengths of the egg extracts were as 1:5:25. The weakest gave a 70-second agglutination.

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smaller than that of sperm that have been in sea water, the fertilizing power of the latter is soonest lost. Schücking reports an experiment of this kind. "Wenn einer grösseren Spermienmenge ein geringes Quantum Eisubstanz zugesetzt war, so dass die Samenfäden nur gelähmt wurden, so konnten die Spermien noch nach 12 Stunden durch Zusatz von Seewasser wieder beweglich und befruchtungsfähig gemacht werden, während die in Seewasser gebrachten Spermien je nach der Temperatur nach fünf bis acht Stunden abgestorben waren" (Schücking, A., 1903, p. 59).

Since the length of time that "egg water" preserves the life of sperm depends upon the relative concentration of the "egg water" and of the sperm, and since the ability of "egg water" to preserve the life of the sperm depends on the decreased activity of the sperm that follows the initial activation, the sooner the sperm become non-motile (or exhibit decreased activity) the longer the span of their life. It is therefore obvious that a very concentrated "egg water" will often be less effective in preserving their life than a less concentrated "egg water." In Table XV. such conditions evidently obtained. Distilled water extracts of eggs (made isotonic by the addition of sea water that had been concentrated by evaporation) such as were employed by Schücking were used in Experiments 13 and 19. Egg extracts can be obtained with very great "agglutinating strength."

In the experiments reported in Table XIV. the sperm suspensions were, for the most part, made in weaker egg waters. (The egg waters employed in different experiments were probably not of exactly equivalent concentration. The concentrations reported are only approximate.) Moreover sperm was added to eggs in large volumes of sea water. Under such conditions the length of life of spermatozoa, as measured by the loss of fertilizing power, is relatively short.

A large part of the effect of "egg water" in preserving the life of spermatozoa is attributable to the hydrogen ion concentration of these solutions.[1] Lillie, F. R., remarked: "That the sea water

[1] Schücking, A., also ascribed this property of egg "extracts" to their acidity. The acidity in the case of his distilled water "extract" of Echinid eggs he believed to be due to mono-sodium and mono-calcium phosphate. The acidity in these experiments was due to the carbon dioxide production of the eggs, for the "egg water" of eggs that had stood in sea water for but short periods of time was used.

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which has stood over eggs combines both the effects of an acid, (aggregation) and also an alkali (agglutination) on the spermatozoa" (Lillie, F. R., 1913, p. 549). The acidity is due to the carbon dioxide that the eggs give forth into the sea water together with any other substances that they may secrete. This carbon dioxide may be removed, and the hydrogen ion concentration of the "egg water" decreased. In that case the ability of the "egg water" to preserve the life of the sperm is much decreased, but is still greater than is that of sea water of exactly the same hydrogen ion concentration. An experiment comparing the effects of egg waters and of sea waters of different hydrogen ion concentrations illustrates this relationship.

Table XVI.

The Effect of "Egg Water" at Different Hydrogen Ion Concentrations upon the Length of Life of Spermatozoa.

Experiment 217.[1]

Concentration of Suspension.	Time of Insemination.	Age of Sperm.		Egg Water.		Sea Water.	
				CO_2 Tension.		CO_2 Tension.	
				1.1.	0.8.	1.1.	0.3.
				Hydrogen Potential.		Hydrogen Potential	
				7.5.	7.7.	7.5.	8.0.
		Hrs.	Min.	Percentage of Eggs Fertilized when One Drop Sperm Added to Five Drops Eggs in 5 C.c. Sea Water.			
0.1%	2.45	0	0				
	3.00	0	15	100	100	100	98
	5.00	2	15	100	97	85	17
	6.00	3	15	100	96	70	8
	7.00	4	15	100	65	15	0
	8.00	5	15	100	47	14	0
	9.15	6	30	91	13	1	0

That the ability of "egg water" to preserve the life of spermatozoa is greater than is that of sea water of exactly the same hydrogen ion concentration is probably in large measure due to

[1] Preparation of egg water: 3 c.c. of eggs that had been strained into sea water at 10 A.M. were allowed to stand in 75 c.c. of sea water until 12.30 P.M. 70 c.c. of the supernatent fluid were then decanted and divided into two 35 c.c. solutions. The hydrogen potential was 7.5, which corresponds to a CO_2 tension of 1.14 mm. The CO_2 tension of the one solution was slightly decreased by passing room air through it until the hydrogen potential was about 7.65 and the CO_2 tension 0.85 mm.

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the higher carbon dioxide and hydrogen ion concentration that is soon reached as a result of the tremendous activity of spermatozoa in "egg water."[1]

After removal of the carbon dioxide, "egg water" can still agglutinate sperm.[2]

Schücking also found that: "Zwei Tropfen des nicht dialysirten sauren Extracts oder ein bis zwei Tropfen des Rückstandes vom Dialysat, mit einem Tropfen 3 per cent. iger Na_2CO_3 Lösung und fünf Tropfen destillirten Wassers agglutinirten das Sperma noch, obgleich sie neutrale Reaction zeigten" (Schücking, A., 1903, p. 61). Accordingly spermatozoa that are added to egg water, where they are very active, produce a large amount of carbon dioxide. This is also true when spermatozoa are put into alkaline sea water. Unlike the former case, however, the carbon dioxide produced is not so effectively neutralized in the case of egg water, and the acidity of the solution rapidly increases. This is followed, as has been shown, by a decreased activity on the part of spermatozoa and a consequent increase in the length of their life.

The effect of egg water upon the fertilizing power of spermatozoa, is, therefore, not so very dissimilar to the effect of the other

[1] The writer is aware of several other possible interpretations of the observation, that (*a*) the ability of "egg water" to preserve the life of the sperm is greater than is sea water of exactly the same hydrogen ion concentration and that (*b*) a very concentrated "egg water" will often be less effective in preserving life than a less concentrated "egg water." For if the "agglutinating substance" be protein in character, as the investigation of Schücking (Schücking, A., 1903) (who found the "agglutinating substance" in the undializable fraction of an egg extract) and of Richards & Woodward (Richards, A., and Woodward, A. E., 1915) suggests the "Studies in the Fertilization of the Eggs of a Sea Urchin (*Strongylocentrotus purpuratus*) by Blood-Sera, Sperm, Sperm-Extract, and other Fertilizing Agents" of T. B. Robertson (1912) may possibly explain these observations. He observed that the "potency of the serum" in initiating either the cytolysis or the development of the egg "obtains a maximum at a dilution of about 1/16" and that "serum of this dilution frequently agglutinates the eggs, causing them to aggregate in large clumps within a few seconds" (Robertson, T. B., 1912, p. 71). In higher concentration the proteins inhibit the imbibition of water by the eggs.

[2] That the "agglutination" phenomenon is not independent of the hydrogen ion concentration of the solution has been shown by Loeb. In acid solution where the sperm are inactive, the "agglutination" cannot occur. The "agglutination" is strongest in solutions slightly more acid than is ordinary sea water but "the clusters" disappear the more rapidly the more alkaline the solution.

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substances that have been studied, and can best be understood by analyzing the effect of egg water upon the physiological condition of spermatozoa. That spermatozoa are activated to an exceptional degree by a secretion from the egg has clearly been demonstrated by the work of all of the investigators who have been quoted. It may be that the fertilizing power of spermatozoa that are added to eggs in sea water during this period of activation is increased in much the same way that the fertilizing power of spermatozoa is increased for a short period of time by decreasing the hydrogen ion concentration (increasing the alkalinity) of sea water. If the sperm are added to eggs after they have become inactive, the percentage of eggs that are fertilized depends upon the degree of inactivity and the degree of reactivation (such as is brought about by a transfer to sea water without a high carbon dioxide tension) which the experimental conditions afford. The sooner the sperm become inactive and the more completely inactive they become the longer their life. Thus, excepting for the initial period of activation, the effect of "egg water" upon the length of life of the spermatozoön is essentially that of a solution of optimal hydrogen ion concentration. The hydrogen ion concentration of the "egg waters" that is most effective in preserving the life of the sperm is precisely that of the acidified sea water that best subserves the same function. In the "egg water" this hydrogen ion concentration is reached as a result either of the carbon dioxide produced by eggs and eliminated into the "egg water" or that produced by spermatozoa during the period of their greatest activity.

XII. Summary.

1. The total available energy of spermatozoa, as measured by total carbon dioxide production, is a constant.

2. The rate at which the total available energy is expended is a function of the activity and an inverse function of the length of life of spermatozoa.

3. The activity and the length of life of spermatozoa is a function of the temperature, and of the hydrogen ion concentration of the suspension. Up to a certain point decreasing the temperature or increasing the hydrogen ion concentration decreases the

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activity and increases the length of life of spermatozoa. Lower temperatures or greater acidities destroy spermatozoa, and the more so the longer they are subjected to these abnormal media. Conversely, within limits, decreasing the hydrogen ion concentration or increasing the temperature increases the activity and decreases the length of life of spermatozoa. Further increase in temperature or in alkalinity irreversibly agglutinates spermatozoa.

4. The ability of spermatozoa to fertilize eggs of the same species is a function of their activity (as measured by their carbon dioxide production, or its converse, their length of life). Spermatozoa lose their power to fertilize as a function of the time of insemination, and of the dilution and of the hydrogen ion concentration of the suspension. If spermatozoa that have been in the suspension for but a short time are added to eggs in a large volume of sea water a decrease in the hydrogen ion concentration of the sperm suspension (that is, an increase in the alkalinity) increases the activity and the fertilizing power of the spermatozoa. Such spermatozoa will, however, lose their power to fertilize when transferred to ripe eggs in sea water long before spermatozoa that have been relatively inactive in more acid sperm suspensions.

5. If spermatozoa that have been at the same hydrogen ion concentration are added to eggs in sea water at the same time, increasing the concentration of spermatozoa increases the percentage of eggs that are fertilized. This may be explained either by assuming that in the greater concentration more spermatozoa will on the average arrive at the periphery of the egg with sufficient action (having the dimensions of energy $\times$ time) to initiate development, or by assuming that mass action of spermatozoa may be effective in initiating the development of the sea urchin egg. The latter explanation has been suggested by Glaser, who pointed out that the further assumption that more than one spermatozoön was necessary "to carry out the bi-parental effect" was not involved.

6. Increasing the carbon dioxide concentration increases the hydrogen ion concentration of the suspension, decreases the activity of spermatozoa and increases the length of their life.

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Sea water of the optimum carbon dioxide concentration for increasing the length of life of spermatozoa is also of the optimum hydrogen ion concentration. It is possible, however, that carbon dioxide affects the physiological condition of spermatozoa otherwise than by means of ionized hydrogen.

7. That decreasing the oxygen concentration of sea water or decreasing the oxidations of spermatozoa by adding KCN to sea water increases the length of their life has been reported by Drzewina and Bohn. Experiments confirming their results and demonstrating that under such conditions sperm are relatively inactive are reported.

8. Spermatozoa increase the carbon dioxide and, therefore, the hydrogen ion concentration of concentrated sperm suspensions to the optimum for decreasing their own activity in a very short time. In such suspensions sperm live for a very long time. McClendon has recently estimated that respiration in sea water that raises the carbon dioxide and the hydrogen ion to a concentration approximately equal to that which is most effective in prolonging the life of a sperm suspension, uses up all of the available oxygen. It is therefore suggested that high carbon dioxide and hydrogen ion concentration and low oxygen concentration are the environmental conditions that obtain in a concentrated sperm suspension. These conditions approximate those of ripe sperm in the testes very much more closely than they do those of sperm that are shed in the ocean.

9. The addition of beef broth to sea water increases the length of life of the spermatozoa. Gemmill suggested that beef broth furnished "artificial nutrition." It is pointed out that the addition of beef broth to sea water increases the hydrogen ion concentration and decreases the activity of spermatozoa.

10. Sea water that has contained the eggs of the sea urchin, *Arbacia*, tremendously activates spermatozoa. It may be that the fertilizing power of spermatozoa that are added to eggs in sea water during this period of activation is increased in much the same way that the fertilizing power of spermatozoa is increased for a short period of time by decreasing the hydrogen ion concentration (increasing the alkalinity) of sea water.

Subsequent to the period of activation spermatozoa become

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inactive in egg water. Such egg water has a high hydrogen ion concentration. This is reached as a result either of the carbon dioxide produced by eggs and eliminated into the sea water, or by spermatozoa during the period of their greatest activity. If spermatozoa are added after they have become inactive, the percentage of eggs that are fertilized depends upon the degree of inactivity and the degree of reactivation (such as is brought about by transferring spermatozoa to sea water without high hydrogen ion or carbon dioxide concentration) which the experimental conditions afford. The sooner spermatozoa become inactive and the more completely inactive they become, the longer their life. The hydrogen ion concentration of egg water that is most effective in preserving the life of spermatozoa is precisely that of acidified sea water that best subserves the same function.

Other effects of egg water upon the physiological condition of spermatozoa have not been considered in this investigation.

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BIOLOGICAL BULLETIN

CRITICAL NOTES ON THE PRESENT STATUS OF THE LENS-PROBLEM.

E. I. WERBER.

1. Introduction.

The present communication is an attempt to arrive at a common understanding of the nature of the difficulties that stand in the way of the solution of a problem to which for over a quarter of a century have been devoted the best efforts of a number of biological observers.

One phase of this problem, although at one time rather near solution, has since, owing to—apparently unavoidable—technical difficulties of the experimental method, become ever more complex. In the following it is my intention to show that this complexity is an artificial one and that it practically disappears on careful sifting of evidence. Owing to this seeming complexity, however, an originally correct interpretation has lately been giving way to one that not only is unwarranted, but has already occasioned even some very improbable and fruitless phylogenetic speculations (cf. for instance Becher, '12).

Anticipating to return to the subject of this communication in a more comprehensive publication in the near future I hope I may be pardoned for incompleteness in considering the pertinent literature at the present time. Only such contributions can here be considered, as from my point of view seem to have the most significant bearing on the problem.

2. The Origin of the Primary Lens—in Ontogeny.

As well known Spemann ('01, '03) was the first to furnish experimental evidence for the correctness of the opinion—advanced simultaneously also by Herbst ('01)—that the lens of the vertebrate eye is dependent in its origin and differentiation

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upon the contact of the optic vesicle with the supra-ocular epidermis from which it arises. It will be recalled also that this view was contested by Mencl ('03, '08), King ('05) and Stockard ('10) and that Spemann himself has in later papers ('08, '12) modified his opinion and abandoned his generalizations. He now thinks that more extended experiments in which various methods were employed on several frog species prove that in some species the lens cannot develop if the optic vesicle fails to come into contact with the overlying epidermis while in one species the epidermis is capable of giving rise to lentiform bodies and even well-differentiated, unmistakable lenses without such contact.

Such conditions would, indeed, be so contrary to expectation that it well behooves us to inquire into the validity of Spemann's self-contesting evidence as well, as of the evidence brought forth by Mencl, King and Stockard for the independent development of the lens.

I have already (Werber '16*c*) pointed out some possible sources of error which would invalidate the conclusions of these authors and on re-reading the entire series of Spemann's interesting papers on the subject I have been struck by the complete absence of any real counter-evidence against the conclusions of his earlier ('01, '03) excellent work.

Spemann's first experiments were performed on *Rana fusca* and consisted in destroying, by pricking with a heated needle or the galvanocauter, the right (presumable) optic pit (foveola optica).[1] As a result he found that no lens was formed, if the entire optic pit had been destroyed or, if deeply buried remnants of the latter which developed into diminutive optic cups failed to reach the epidermis. If, however, such a rudimentary optic cup did come into contact with the epidermis, a lens developed secondarily from the latter.

I am inclined to think that these results practically solved the problem, and for this reason it may perhaps be regarded as unfortunate that Spemann's well-warranted generalizations have been the subject of ill-founded criticism by Mencl ('03). For while the latter was very aptly met by Spemann ('03), it ap-

[1] For a description of this earliest discernible eye primordium cf. Eycleshymer ('93 and '95) and Froriep ('06).

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parently occasioned him to enter upon a great many experiments on the subject which, though beautifully conceived and skillfully executed, have, owing to the pitfalls of a treacherous material, yielded fallacious results.

The first experiments which have occasioned Spemann ('07) to modify his views were performed on *Rana esculenta*. They consisted in the excision of the "right anterior half of the brain primordium" from the wide open medullary plate.[1] As a result he observed that a lens has developed in spite of the absence of an eye on the side operated upon. Like results were obtained also when the optic pit was destroyed at the same stage of development with a heated needle. The results were apparently entirely independent of the method of operation. In both cases, however, they differed from those obtained in *Rana fusca*, in which, as we have seen, no lens was formed when the optic anlage was at this stage destroyed by pricking with a heated needle, while the excision experiment in this species, according to Spemann ('07) "ergab ein sehr unsicheres Resultat." In one out of four examined embryos Spemann ('12) observed "ein kleines Bläschen, welches eine Linsenanlage sein könnte."

Why such divergent results in experiments on two species of the same frog genus with the same methods and at the same stage of development? Spemann concludes from his experiments on *Rana esculenta* that in this species the "lens-forming cells" of the embryonic epidermis are capable of independent development in the absence of an optic vesicle, while *Rana fusca* lacks this ability for self-differentiation.

The same experiments on *Bombinator pachypus* (at the same stage of development) gave less definite results. While no lens developed in this species on the side operated upon, Spemann observed in some few instances structures which he could not with certainty identify. Experiments on embryos of the same species at a later stage of development, *i. e.*, removal of the optic vesicle, the overlying epidermis being previously raised up and reflected from it, gave also ill-pronounced results. In a number

[1] In my 1916c paper several, otherwise wholly insignificant, errors have unfortunately crept in into the references made to these excision experiments in *Rana esculenta* and to King's pricking experiments. In both cases it should read "optic pit" or "optic anlage" instead of "optic vesicle."

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of instances Spemann observed in such embryos on the side operated upon a thickening of the epidermis in the region where a lens normally should have developed, which he is inclined to consider as an abortive attempt at lens-formation. From these experiments Spemann tentatively concludes that *Bombinator* in regard to the power of independent lens-formation occupies a sort of intermediate position between *Rana fusca* which lacks this ability and *Rana esculenta* which, according to him, possesses it in a decidedly marked degree.

Are these conclusions justified? I am strongly inclined to think that they are not, the results on which they are based being very inconclusive. Moreover, on careful examination of the excision experiments in *Rana esculenta* the impression is gained that some fragments of optic substance were left after the excision of the anterior half of the primordium of the brain hemisphere. Thus, for instance, in his 1912 paper in Fig. 6 the smaller one of the two observed lentoids is in close apposition to a "ganglion." May not this ganglion rather be a small group of retinal cells? In Figs. 8 and 9 small groups of tapetum nigrum cells may be observed, and Spemann (p. 22) admits "dass vom Augenbecher vielleicht kleine Reste vorhanden seien." In Fig. 8 the tapetum-cells are very close to the lentoid, while Fig. 7 also suggests that dispersed tapetum nigrum was responsible for the origin of its "Linsenbläschen." Instead of concluding, as does Spemann, that the lentoids of Figs. 6 and 9 are due to dispersion of "lens-forming cells" it might perhaps be safer to think that in these experiments fragments of potential optic-cup substance were dispersed and stimulated the differentiation of lentoids by chancing to come into contact with the epidermis.

Again, in Fig. 10 representing a cross-section through an embryo in which the anterior part of the anlage of the right brain hemisphere has been destroyed by a heated needle, the lentoid observed is in close proximity to a rudimentary optic cup (oc′, Fig. 10), which clearly demonstrates the fact that the attempted elimination of the anterior part of the brain primordium may often be illusory, fragments of optic-pit substance remaining after the operation. According to Spemann ('12, pp. 24 and 25) it is easier to eliminate the entire optic anlage in *Rana fusca* than

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in *R. esculenta*. He attributes this to a difference in the "Konsistenz" between the embryos of the two species. "So ist wohl das Resultat zu erklären, dass der Defekt bei den allerdings nicht sehr zahlreichen Versuchen dieser Art meist entweder zu gross wurde, im ersteren Fall also vom rechten Auge ein Rest erhalten blieb, im letzteren die Linsenanlage mit zerstört wurde."

In my estimation the divergent results obtained both by pricking with a heated needle and by excision in both species would rather seem to point to differences in the location of the optic pits in the neurulae of the two species at the same stage of development. Thus, in *Rana esculenta* they are probably more diffuse and extend more laterally than they are in *R. fusca*. Accordingly, if at this stage the excision of the anterior half of the brain primordium be attempted, it may happen that a small fragment of the optic pit may remain, as in this species it may extend even laterally from the medullary fold. Granting, however, that fragments of potential optic-cup substance do sometimes remain after the operation in this species—and Spemann admits that both in text and in the figures—it is no longer difficult to understand that in such embryos lentoid structures and even well-differentiated lenses may be formed on the eyeless side owing to the "lentogenic stimulus"[1] from the remnant of optic substance on the epidermis.

Spemann does not seem to have considered the results obtained from these operations as very conclusive, if as much can remain of the brain anlage after its attempted elimination as "ein Fragment des Tapetum nigrum," "der vorderste Teil der Vorderhirnanlage, . . . ein dorsales Stückchen Zwischenhirn mit Epiphyse und Plexus chorioideus, jedenfalls entstanden aus lateralen Partien der Medullarplatte, endlich ein Stückchen der linken Hälfte des Mittelhirns" ('12 p. 28). For, not content with these results he sought confirmation in experiments in which several other methods were employed.

Thus, adopting Lewis's ('04) method, he removed (by excision) in *R. esculenta* the optic vesicle after having previously raised up and reflected the overlying epidermis. The latter was then affixed in its place where it healed and in two embryos gave rise

[1] Cf. Werber '16c.

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to "ein deutliches Linsenbläschen" in the absence of the optic cup. Spemann considers these lens-buds as evidence of the ability for independent lens formation in *R. esculenta*.

The validity of this conclusion, however, seems questionable to me in view of a well-nigh uncontrollable source of error which Spemann himself has pointed out ('12, p. 42). For he states expressly that the lower stratum of the epidermis (the "Sinnesschicht") adheres so firmly to the eye vesicle that on attempting to separate the epidermis from the latter usually the upper layer (the "Deckschicht") is raised up, while the lower one remains attached to the optic vesicle. Regardless of Spemann's skill in such delicate operations and the precautions he has taken to separate the entire epidermis from the optic vesicle it was apparently impossible always to avoid minute fragments of the latter remaining attached to it. And the two cases in which he obtained "independent" lens-buds have possibly resulted from just such an unsuccessful operation, while in the embryos in which no such structures were recorded on the side operated upon the operation was apparently faultless.

Spemann assumes that in his unsuccessful experiments of this series, *i. e.*, where no "independent lenses" were recorded, the "lens-forming cells" of the lower stratum of the epidermis were removed with the optic vesicle to which they remained attached. I can see no valid reason for the assumption of cells predetermined to form lenses. The often-raised argument of the cyclopean eye in which the lens is formed from epidermis that normally does not develop into this structure, and Lewis's ('04, '07a and b) experiments in which the formation of lenses from strange ectoderm and even from ectoderm of another species was demonstrated to be possible—an experiment successfully repeated by Spemann himself by transplanting the bared optic vesicle of *Rana esculenta* under the ventro-abdominal epidermis of *Bombinator*—speak very decidedly against this assumption.[1]

Moreover, why should just the species *Rana esculenta* form such a strange exception as to possess such so early specialized

[1] Mencl ('08, Fig. 3) described an "intracerebral eye" (intracerebral cyclopia) whose lens is derived from the epithelium of the mouth. The ability of this part of the ectoderm to give the "lentogenic reaction" is demonstrated also by the lentoids of the mouth which I have recorded (Werber '16c).

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regions of the epidermis? I am under the impression that the apparent fallacy of Spemann's conclusions from this series of experiments is most likely due to the circumstance that while he detected an important source of error, he unfortunately, mistook the nature of the error. For had he considered the possibility that fragments of the optic vesicle too small to be detected with the binocular dissecting microscope and even so minute as not to be able to differentiate into histologically discernible structures, may suffice to induce the lentogenic reaction in the epidermis to which they remained attached, he would probably have considered the "unsuccessful" experiments of this series as successful and vice versa. Accordingly, instead of believing that in the two "successful" cases the "lens-forming cells" had not (or not entirely) been removed, he might have concluded that owing to the above-mentioned difficulty some remnants of optic substance have stimulated the development of the lens-buds demonstrated in Figs. 37, 38 and 38a.

The results of other experiments performed by Spemann ('12) which may now be examined would also seem to lend support to his former views rather than to his present ones. One of these experiments consisted in transplanting abdominal epidermis over the bared optic vesicle in *Rana esculenta*. The flap of epidermis used for the purpose was previous to its transplantation "von etwa anhaftenden Mesodermzellen sorgfältig gereinigt." The result was negative—no lenses or lens-like structures were formed by the strange epidermis owing, as Spemann thinks, to the circumstance that the "primären Linsenbildungszellen" were contained in the flap of supra-ocular epidermis which had been removed. However, four cases were recorded in which the epidermis transplanted over the eye presented an appearance different from that of the epidermis of the immediate surroundings. And in Fig. 63 there is "eine deutliche Linsenanlage (L) vorhanden, ein kleines, dickwandiges Epithelbläschen, welches mit der transplantierten Rumpfhaut über dem Auge innig verbunden ist." ('12, p. 55). Spemann mentions also another embryo of this series in which he observed an indication of lens-formation on the side operated upon (Fig. 58), but assumes that in both cases "primäre Linsenbildungszellen" remained attached

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to the optic vesicle owing to faulty technique in the removal of the supra-ocular epidermis.

Might we not with at least just as much justification assume that in the embryos in which no lenses were formed from the transplanted epidermis this was due to lack of immediate contact between the optic vesicle and the epidermis, because mesodermal cells were attached to the latter? For, regardless of the most painstaking care I think it is impossible to be sure that the epidermis has been cleaned of these cells entirely with the aid of such low magnifications as even the highest power lenses of the binocular dissecting microscope. It is, besides, difficult to understand why the optic vesicle of this species should be able to induce lens-formation from ventro-abdominal epidermis of *Bombinator* and not from the abdominal epidermis of an embryo of its own species.

Whatever one might think of the results of these experiments, they certainly cannot be regarded as conclusive in favor of Spemann's present views.

Two more series of experiments on *Rana esculenta* may now be considered the results of which Spemann interprets in favor of the independent development of the lens in this species. The method employed in these experiments was autoplastic transplantation and the part transplanted was the "primären Linsenbildungszellen."

In the first (a) series a more or less rectangular piece of supra-ocular epidermis was detached during or immediately after the closure of the medullary tube and turned about 180°, care being taken that no fragments of the optic vesicle be left on it. In the second (b) series the operation differed only in one point, viz., that in detaching the supra-ocular epidermis a fragment of the optic vesicle remained attached to it and owing to the inversion of the piece of epidermis (by turning it about 180°), it became transplanted into a posterior region (otic capsule).

In the experiments of the first (a) series great technical difficulty was encountered and only in four cases was the separation of the epidermis from the optic vesicle "anscheined einwandsfrei." In three of these cases the eye of the operated side had "eine deutliche Linse," while in the fourth case where, owing to

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the operation, the otic labyrinth came to lie between the epidermis and the eye, the latter lacked a lens. No lens, however, developed in any of these embryos caudally from the eye, as might have been expected, had that part of the epidermis ("primäre Linsenbildungszellen") been capable of giving rise to a lens by self-differentiation.

These cases (although so few in number) point decidedly, I think, to the inability of supra-ocular epidermis in *R. esculenta* to differentiate a lens in the absence of a stimulus from the eye vesicle. Spemann, however, prefers the tentative explanation "dass nach Auslösung einer Linsenbildung durch's Auge die spontane Entwicklung einer zweiten Linse aus den primären Linsenbildungzsellen unterbleibt." He is inclined to consider even the possibility "dass bei diesen Experimenten die Linsenbildungszellen nicht weit genug nach hinten gebracht worden waren und dass entweder sie selbst oder ihre nächste Umgebung die Linse des stehengebliebenen Auges geliefert haben." As support for the latter possibility he regards the observation, "dass die Linse in allen 3 Fällen im hintersten Winkel des etwas deformierten Augenbechers liegt und einmal sogar etwas in die Länge gezogen ist" ('12, p. 64). Is it not more probable that this distorted relation between the lens and the optic cup is due to the very fact that the latter was deformed owing to the operation? From my observations on many teratophthalmic *Fundulus* embryos I know that this is very often the case in deformed eyes.

One cannot escape the impression that Spemann by considering such possibilities and by regarding "diese Versuche . . . als misslungen und die Frage als unentschieden" has—apparently unconsciously—missed the only obvious conclusion, viz., that *Rana esculenta* possesses no early predetermined lens-forming cells and that a stimulus from an optic cup or from some of its parts is necessary to induce the differentiation of a lens from the epidermis in embryos of this species as much as in *Rana fusca*.

Let us now consider the second (b) series of these experiments in which, as will be recalled, a fragment (the tip, "Kuppe") of the optic vesicle was left attached to the inverted epidermis.

Observations were made on seven embryos operated upon in

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this manner. In all of them the anterior eye fragment lacks the lens, while the posterior one in five embryos stimulated the formation of a lens which "*kann* so vollkommen entwickelt sein wie die normale" (p. 67). In one of these embryos the posterior eye fragment possesses even two lenses which Spemann is inclined to regard as being due to mechanical separation of the "Linsenmaterial" into two parts, although it would seem more probable that the duplication of the lens in this case may (owing to the operation) be due to mechanical histolysis of the eye fragment which thus came into contact with the overlying epidermis in two places.

From these observations Spemann (p. 67) concludes that "die weitgehende Determination der primären Linsenbildungszellen von *Rana esculenta*, welche sie zu selbstständiger Entwicklung befähigt, bringt es wohl mit sich, dass die übrigen Epithelzellen, auch die der nächsten Umgebung, nicht mehr imstande sind, auf einen Reiz des Augenbechers mit Linsenbildung zu antworten."

These conclusions cannot be considered as warrantable. Moreover, the results permit of an interpretation which, I believe, is founded upon a much greater probability. It must be remembered that at the stage of development at which the operations were performed, it is only the tip ("die Kuppe") of the eye vesicle that is protruding from the head. If that protruding part be removed, the epidermis transplanted over the resulting gap may, owing to the latter, fail to come into contact with the deeper part of the eye even on subsequent growth of the latter owing to the obstruction formed by mesenchyme cells which may grow into the gap before such contact can be effected. Spemann's Fig. 67*b* very forcibly suggests just this possibility. On examining the anterior eye fragment of this figure one can observe that it is not in contact with the epidermis. More significant, however, is the observation in its lumen of a blood vessel and of some mesenchyme cells which also fill the space between it and the epidermis.

Our interpretation of these apparently vexing cases receives further support from the very fact that in the (a) series of these experiments where the "Kuppe" of the optic vesicle had not

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been removed, the latter was able to stimulate the formation of lenses, because there being no gap between it and the epidermis transplanted over it, there was no chance for an obstruction of its contact with the latter by ingrowing mesenchyme. It is, besides, needless to say that it is difficult to understand just why Spemann in the experiments in which the epidermis was transplanted over a defective eye vesicle assumes a sharp predetermination to form lenses and not so, if transplanted over an uninjured eye vesicle.

Summarizing briefly *we find that critical sifting of the results of Spemann's experiments on Rana esculenta discloses no warrantable evidence whatsoever for the presence in this frog species of an early predetermined lens-forming part of the epidermis capable of differentiation into a lens without the stimulus from an optic vesicle or at least a fragment of it.*

Practically all of these experiments were performed by Spemann also on *Bombinator pachypus*. The results obtained in this species differed according to Spemann ('07, '12 ff) from those obtained in the pricking experiments in *Rana fusca* and also from the above-noted results in *Rana esculenta*. They indicate, Spemann believes, that *Bombinator* in regard to the ability to form a lens by self-differentiation occupies a position intermediate between *Rana fusca* and *Rana esculenta*.

It is evident, I believe, that were this really so, *i. e.*, if the supra-ocular epidermis of *Bombinator* possessed a certain degree of this ability and only needed the stimulus from the optic vesicle as a complementary aid for the differentiation of a lens, a serious stumbling block would here confront every attempt at the solution of the lens-problem.

Fortunately, however, Spemann's observations seem to call for an entirely different interpretation of the results.

Thus in the excision experiment (right foveola optica) the latter prove very decidedly, I believe, that no lens can be formed in the absence of an optic anlage or on its failure to come tinto contact with the overlying epidermis. For, out of the forty-six embryos operated upon in twenty of them in which an optic fragment remained (owing to incomplete excision), a lens developed on the side of operation. Of the remaining twenty-six

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cases one is uncertain, for here a structure was observed which might perhaps be a deformed lens-bud, in twenty cases neither an optic cup nor a lens can be observed, while in five cases the optic cup is so small that there was no contact with the epidermis. According to Spemann, however, in two of the latter cases the condition is not clear, one of the embryos possessing (on the side of excision) near the brain "ein dickwandiges Bläschen mit kleinem Lumen," which Spemann is unable to identify, while in the other case the region of epidermis which should have furnished the lens, is "kaum merklich verdickt."

The only warranted conclusion from these results is: *The contact of at least a fragment of the optic anlage is necessary to induce the formation of a lens from the overlying epidermis.* It is unwarranted however, to conclude, as does Spemann (12, p. 38), ". . . dass Bombinator pachypus zwar auch primäre Linsenbildungszellen besitzt, welche von den Epidermiszellen der Umgebung verschieden und zur Umbildung in die Linse vorbereitet sind, dass diese Zellen aber der Mitwirkung des Augenbechers bedürfen, um in Aktion zu treten, zum mindesten in viel höherem Masse als die Linsenbildungszellen von R. esculenta."

The results of other experiments in this species also point decidedly to the incorrectness of Spemann's conclusions. Thus the removal of the optic vesicle during or immediately after the closure of the medullary folds resulted only in a thickening ("Wucherung") of the epithelium in the region where normally the lens should have arisen. In two cases, however, small remnants of the optic vesicle developed into diminutive optic cups lacking a lens, because mesenchyme had grown in between them and the epidermis. Lentoid structures (two) were observed only in one case (Figs. 41*a* and *b*, L′), which are probably due to a stimulus from very minute remnants of the optic vesicle.

These experiments would again seem to *prove only that*, as Lewis ('04, '07*a* and *b*) and Le Cron ('07) have found, *a stimulus from the optic vesicle on the epidermis of sufficient duration is necessary for the latter to give rise to a lens.* They do not prove, however, the presence in this species of early predetermined lens-forming cells requiring the stimulus from an optic vesicle only as a supplement for full differentiation into a lens.

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On transplanting ventro-abdominal epidermis over the bared optic vesicle in this species Spemann recorded as a result that in none of the sixteen examined embryos a lens developed from the strange epidermis. From these results he concludes that either the optic vesicle cannot stimulate abdominal epidermis to the formation of a lens or that this part of the epidermis is incapable of responding to such a stimulus.

Here again, however, the same objection is unavoidable which we have raised against the conclusion from the results of the same experiments in *Rana esculenta*, namely that regardless of the experimenter's care mesenchyme cells attached to the transplanted epidermis obstructed a contact of the latter with the optic vesicle.[1]

Very clear ("eindeutig") results were obtained in the experiments in which, as in *Rana esculenta*, a flap of the skin of the head including the supra-ocular epidermis was turned about 180°. In the first (a) series in four out of eight "einwandsfreie" cases the eye cup was not in contact with the epidermis and, naturally, lacked a lens. In the other four cases there was such contact—and the eye possessed a lens.

Accordingly: *There are no lens-forming cells, but epidermis that normally does not give rise to a lens will differentiate into a lens, if brought into contact with the optic vesicle.*

The second (b) series of these experiments (a fragment, the "Kuppe," of the optic vesicle transplanted with the turned epidermis) yielded results very similar to those of the corresponding experiments in *Rana esculenta*.

With but one exception the posterior eye fragment always obtained a lens, which, as Spemann rightly adds, could not have developed in the absence of this fragment of optic-cup substance.

The anterior eye fragment, however, obtained no lens in fifteen out of the twenty examined embryos, while a lens or only a thickening of the epidermis opposite the eye fragment was ob-

[1] It is significant that in one out of the four cases in which he reports to have observed a "Wucherung" opposite the eye "eine zusammenhängende Schicht sehr dotterreicher Zellen" (p. 60) was conspicuous in that interspace. That shows that it is apparently futile to attempt to clean the abdominal epidermis of all mesenchyme cells. While it is difficult to know just how to interpret these "Wucherungen," they might possibly be due to mechanical distortion during the operation.

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served in five cases. In thirteen of the embryos which had no anterior lens Spemann accounts for its lack by the ingrowth of mesenchyme into the space between the optic-vesicle fragment and the epidermis, widened by the expansive growth of the otic capsule which, owing to the transplantation came to occupy this anterior position. He is unable, however, to suggest an explanation for the lack of this lens in two cases, "wo kein Grund für ihr Ausbleiben zu erkennen ist" (p. 73).

From these experiments Spemann ('12, p. 77) concludes that the optic vesicle of *Bombinator* is capable of exerting a specific stimulus not only on the lens-forming cells, but also on other parts of the head epidermis.

This conclusion is, I think, only partly correct, the assumption of "primäre Linsenbildungszellen" being both unwarranted and unnecessary. For leaving out of consideration the two cases (which I should not wish to pre-judge), where no anterior lens was formed while it should have been expected, we can conclude only that its presence in the several other cases was due to the specific stimulus from the anterior eye fragment.

Briefly, the results of this series of experiments also contradict Spemann's opinion of the intermediate position of *Bombinator* between *Rana fusca* and *R. esculenta* with regard to the ability of independent differentiation of the lens.

In *all of Spemann's work* I find only *a confirmation of his initial results and strong support for the generalizations which he made in 1901 and 1903, but no counter-evidence whatsoever that would justify his present ('12) opinion.*

Other evidence for the independent development of the lens brought forth by Mencl ('03, '08), King ('05) and Stockard ('10) is no less illusory.

Mencl ('03) described the head of an anophthalmic component of an anadidymus in *Salmo salar* which possessed two laterally located lenses although there could be observed "keine Spuren von Augenblasen, ja nicht einmal von Anlagen derselben." Both of these lenses were in close apposition to the deformed brain, the larger one even having, owing to its growth, formed a pit in the latter by pressure. Both lenses, however, were so situated that their derivation from lateral parts of the head

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epidermis was beyond doubt. What is responsible for the origin of these lenses? Mencl suggested (p. 337) that "der, diese zwecklose, wie durch Erinnerung der Epidermiszellen auftauchende Linsenbildung auslösende Faktor ist die Vererbung."

A much more reasonable and, I think, the only correct, interpretation of this case was offered by Spemann ('03) who concluded (p. 464) that the optic vesicles "oder genauer ihr für die Linsenbildung allein in Betracht kommender retinaler Teil nur scheinbar fehlen, indem die Partie der Hirhwand, welcher die Linsen angelagert sind, nichts anderes ist, als die nicht abgegliederte . . . Retina."

Although this interpretation was later abandoned by Spemann "unter dem Druck neuer Tatsachen" ('12, p. 3), my experience with teratological material—and Mencl's case is a teratological one—forces me to lend it unreserved support.

For the sake of clearness in presenting my point of view (based largely on teratological data) the following brief recapitulation of the present stage of our knowledge on the morphogenesis of monsters may be permitted.

It was repeatedly noted by pathologists and teratologists and notably also by Mall ('09) that examination of malformed embryos often discloses evidence of destruction, dissociation and shifting of tissues or parts of the embryo. On examination of a great many experimentally produced monsters in *Fundulus heteroclitus* I ('16*a* and *b*) was able not only to confirm these observations, but also to demonstrate some remarkably striking cases of the effects of such dissociation of parts of the early embryonic primordium and to account for the causes of this process which I have termed blastolysis. This "blastolytic action of the chemically modified environment is . . . a morphogenetic principle common to all terata. . . . Blastolysis either destroys part or all of the germ's substance, or it may split off and disperse parts of the latter" (Werber '16*b*, p. 569).

It is this destruction of tissues, the subsequent elimination of parts destroyed and the resulting dissociation and shifting of parts surviving that in experiments in which eggs are subjected to a chemical modification of the environment, brings about the weirdest malformations of the developing embryos.

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All of these deformities are thus clearly *due to a defect (or defects) of a blastolytic nature.* Not only is this the case in experiments in which the modification of the environment employed is a chemical one, but also, if a physical, *e. g.*, thermic, modification be employed. For, in experiments (not yet published), in which the eggs of *Fundulus heteroclitus* were subjected to the action of a temperature much below the normal, some terata and particularly ophthalmic monsters resulted. The latter are due to loss at an early embryonic stage of parts of ophthalmoblastic material and to like damage sustained by the anterior part of the potential head. Thus anophthalmia results from such loss by the earliest brain primordium of the whole or nearly whole ophthalmoblastic material.

In some anophthalmic embryos such blastolytic optic-cup fragments or even small, fairly well differentiated optic-cups may on microscopic examination of sections be observed either enclosed in the brain between both hemispheres (intracerebral cyclopia, as it were, cf. Mencl '08, Figs. 2 and 3) or in a lateral position as a part of the brain. Such "concealed" remnants of the optic cup may often lack the tapetum nigrum and the histological character of retina, owing to early destruction of some groups of cells which potentially corresponded to the lacking layers of the retina, and may thus give the appearance of a part of the brain. If it happens to come into contact with the epidermis, such "a part of the brain" will stimulate the differentiation of a lens from it.

This, undoubtedly, is exactly the condition in the case described by Mencl in 1903. I have many times observed unmistakable optic cups which lacked some of the histological characteristics of retina and whose structure was very much like that part of the "brain" in Mencl's case (cf. Mencl '03, Figs. 2, 4 and 5, and also '08, Fig. 1), into which the larger one of the two lenses has, as it were, burrowed in. That part of the "brain" is undoubtedly a "verkappte" retina, as Spemann had once suggested.

This, I think, disposes of the first, but most troublesome, case described by Mencl. It might perhaps be added that the asymmetric position of the two lenses[1] in the head of this anophthal-

[1] "Die linke liegt mehr dorsal und zugleich caudalwärts von der rechten" (Mencl, '03, p. 331).

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mic anadidymus component could not be accounted for by "hereditary reminiscences," while chance contact of remnants of dissociated ophthalmoblastic material of both sides with the epidermis does account for the origin of both lenses as well as for their asymmetrical position. Neither of them has arisen from predetermined, lens-forming cells—"durch Erinnerung," but from indifferent ectoderm in response to stimuli from remnants of potential optic cups on the epidermis.

The other "independent" lenses and lentoids described by Mencl ('08) can even much easier be demonstrated to be due to the same origin—blastolysis and subsequent contact of fragments of optic cup substance with the ectoderm. From his description of sections of the embryos underlying the Figs. 4, 5, 6, 7 and 8 the reader will easily gather that in these cases fragments of optic-cup substance[1] have been profusely dispersed and in places of contact with ectoderm gave rise to lenses or lentoids. Mencl did not appreciate the full significance of his own observations, for referring to such an optic fragment ("Pigmentanhäufung," Fig. 5*X*, he says: "Was dies Gebilde bedeutet, *kann ich nicht entscheiden—das Vorhandensein des Pigments jedoch, sowie die retinaartige Anordnung der Zellen in der benachbarten Hirnwand*[2] lässt die Meinung entstehen, dass es sich dabei um irgend ein Rudiment einer atypisch und selbstständig zur Entwicklung gelangten Augenkomponente handelt" (p. 447). It is, indeed, difficult to understand just why, in spite of these observations, Mencl claims that in these embryos "die selbstständige Entwicklung der Linsen über jeden Zweifel erhaben ist" (p. 447).

I have recently examined sections of a number of deformed Salmonid embryos (of another species) with such "independent" lenses. The publication of the observations made on this material being reserved for a future publication, it may suffice to state that the conditions noted in several of these embryos bore a very striking resemblance to those in the embryos lately described by Mencl ('08). Here, too, subjective interpretation

[1] "Pigmentanhäufung," "Pigmentbläschen," "Pigmentfleck," "eine homogene, schwarze, längliche Pigmentmasse, welche in die Falte der retina-artigen Hirnwand hineinragt" (Mencl '08, p. 447).

[2] My own italics.

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might easily lead to the conclusion that the "eyeless" lenses arose by self-differentiation of the ectoderm. But careful observation and rigid analysis of the noted relations of parts to each other leaves no doubt that all these lenses are products of stimuli from dispersed optic-cup substance.

In a *Fundulus* monster described by me in a former paper (Werber '16*c*) there were no eye defects, but owing to a special method optic-cup substance was very profusely dissociated and dispersed through a large part of the head. Owing to this condition a great deal of the head ectoderm and even the epithelium of the mouth, infected, as it were, with such fragments of the optic anlage, responded by the formation of a great number of lentoids. Many more monsters resulting from the employment of the same method were examined in sections and they show very similar conditions. In other experiments in which the method employed was more destructive various eye defects such as monophthalmia or synophthalmia or anophthalmia resulted. On examination of sections through these embryos not only lentoids, but well-differentiated, "eyeless" lenses were frequently observed and in nearly every one of these cases some more or less obvious traces of optic-cup substance can be observed in their immediate neighborhood.

In the same paper I have pointed out that the "independent" lenses which Stockard ('09, '10) described in teratophthalmic *Fundulus* embryos have also undoubtedly resulted from contact of ectoderm with such blastolytic fragments of the eye anlage.

On that occasion I have also called attention to a very probable source of error in King's ('05)[1] experiments in view of which the evidence she brought forth for the independent development of the lens in *Rana palustris* in contradiction to Lewis's ('04) unmistakable evidence to the contrary, appears to be illusory.

Experiments of other observers (Bell '06 and '07 and Ekman '14) also yielded results which decidedly contradict the idea of the independent development of the lens. And a very beautiful demonstration of the "lentogenic reaction" of the epidermis to a stimulus from an eye fragment was recently furnished by Wachs ('14, p. 430 and Figs. 46, 47, 48 and 49).

[1] Cf. the footnote on p. 221 of this paper.

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This author transplanted in well-advanced larvæ (hind legs developed) of *Triton tæniatus*, in which the eye was at the time of operation as fully developed as in the adult, a piece of the iris from the eye of one larva into the otic capsule of another larva of the same age. As a result he could in one case observe that a strand of epidermal epithelium which, owing to the operation, had grown in from the edge of the wound to the otic capsule, has given rise to "ein rundes Lentoid . . . mit konzentrisch gelagerten Linsenfasern . . .."

From all that has been said so far we may conclude that *there exists no valid evidence for the possibility of the origin of the lens by self-differentiation.* On the contrary, *observation in the normal development of the eye, evidence from teratophthalmic cases and all experimental evidence point to the correctness of Herbst's ('01) and Spemann's ('01) conclusion that the lens of the vertebrate eye depends in its development and differentiation from ectodermal epithelium upon a specific, apparently chemical, stimulus from the optic vesicle.*

This stimulus might perhaps be in the nature of an enzyme action by contact. Spemann ('05, '12), who recognizes the dependence of the development of the lens upon this specific stimulus in some species, assumes the possibility of a specific secretion by the optic cup or rather by the retinal part of the latter, to which, as he believes, may also be due the "regeneration" of the lens from the iris of the fully developed amphibian eye. While this hypothesis appears to have received strong support from the beautiful experiments of his pupil Wachs (*l. c.*), it seems to me perhaps premature to speak of a secretion in this case. For, aside from other considerations, the fact ascertained by Wachs (*l. c.*) that the supposedly secreted substance is not conveyed by the blood of the animal to any other part of the body would seem sufficient to indicate that the retina, as Wachs himself concludes, has no such endocrine function. Nor is Wachs's assumption of a secretion that "bleibt auf das Auge und seine nächste Umgebung beschränkt" (p. 446) justified by the results of his numerous experiments.

There is, moreover, good reason to believe that the capacity for the "lentogenic stimulus" is not at all restricted to the retinal

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layers of the eye cup. For evidence is not lacking that the tapetum nigrum is also capable of inducing the development of a lens (or lentoid) when in contact with ectodermal epithelium. Thus, as pointed out above (p. 235), some of the "independent" lenses recorded by Mencl ('08) very clearly owe their origin to contact of ectoderm with tapetum nigrum fragments ("Pigmentbläschen," "Pigmentanhäufungen." "Pigmentmasse"). It was also mentioned that in a considerable number of *Fundulus* monsters in my possession "independent" lenses and lentoids can be demonstrated to be due to this "enzyme action" of the tapetum nigrum. This ability of the pigment layer of the optic cup is also suggested by some of Spemann's ('12) and Stockard's (*l. c.*) figures.

Whatever the nature of this action may be, whether or not we agree to regard it as an enzyme action, as I am inclined to do, it seems evident that all layers of the optic cup are capable of it. Even the iris epithelium which genetically is also a part of the optic cup may also be capable of this action.

3. The Origin of the Secondary Lens—in "Regeneration."[1]

Bearing in mind this apparent ability of the embryonic ectoderm for lens formation—by "enzyme action," it may perhaps no longer be difficult to account, at least theoretically, for its secondary formation—"regeneration"—from the iris after the exstirpation of the primary lens from the fully developed eye of the amphibian larva or even the adult.

An attempt in this direction has already been made by Wachs (*l. c.*) who accounts for this phenomenon as a reaction of the epithelium of the iris to the "secretion" of the retina. Although I find it difficult to accept the evidence for this secretion, I believe that Wachs's idea is correct in the main. The secondary for-

[1] I am not inclined to regard the formation of the lens from the epithelium of the iris as a case of true regeneration in the precise meaning of that term. *From the histogenetic point of view* we must say that *this matrix (the iris) gives rise to the lens for the first time* and thus it *generates*, but does *not re*generate, it. *Considering*, on the other hand, the *secondary lens in relation to the whole visual organ or to the entire body of the animal*, it is difficult to deny that *we are here dealing with* a case of what is commonly regarded as *regeneration* ("Ersatz", "Nachbildung", "Nachwachsen", "Wiederwachstum"). Without wishing, therefore, to decide on the fitness of the term in this connection, I am employing it only in quotation marks.

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mation of the lens—from the epithelium of the iris—is probably a response (reaction or complex of reactions) to a chemical stimulus just as much as its primary development from the supra-ocular epidermis of the early embryo.

But, it may well be asked, if there is no such secretion as assumed by Wachs, what is the nature of the chemical stimulus and whence, from what source, or from what part of the eye does it issue? Two possibilities suggest themselves as an answer to this query. It may be imagined that, owing to the operation, the iris comes into temporary contact with the inner wall of the optic cup, from which it might thus receive the stimulus for the formation of a lens in the same manner as in the embryo the supra-ocular epidermis from the optic vesicle. Such contact due to a collapse of the optic cup[1] may result from the methods of operation employed by Wolff ('95) and the other experimenters (with the exception of Wachs). However, I am not inclined to give serious consideration to this possibility, as this contact of the pupillary edge of the iris is not necessary for the "regeneration" of a lens, for in Wachs's experiments the method of operation excludes it altogether. The experiments of the latter author, however, do not, as he believes, exclude another possibility which to me seems well worthy of careful consideration.

Wachs (*l. c.*, pp. 416–426) has raised the question whether the iris is capable of forming a lens without any outside stimulus ("aus sich heraus"). He was led to consider this possibility by the results of some of his experiments, in which a fragment of the iris, implanted into the posterior chamber of an eye deprived of its lens "regenerated" a lens, even if it did not heal on to the iris. This lens may even be better differentiated than the lens simultaneously "regenerated" by the latter.

To decide whether the fragment of iris in these cases formed a lens under the influence of a secretion from the retina or—"aus

[1] According to a statement by Müller ('96) no lens is formed by the iris after extraction of the primary lens, if the vitreous body is injured. Were this statement correct, no lens could arise from the iris owing to its contact with the optic cup by collapse of the latter. It is evident, however, that Müller's observation is incorrect, for in Wolff's (*l. c.*) and in some of Fischel's ('00 and '02) experiments in which a lens or lentoids did form from the iris, the vitreous body was undoubtedly injured, since the whole optic cup was damaged.

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sich heraus," he transplanted (in a number of experiments) a fragment of the iris under the skin of the head. The result was negative, as the piece of iris disintegrated and was resorbed.

Wachs concluded from this result that the site of this transplantation was apparently unfavorable and he, therefore, in the next experiments transplanted a fragment of the iris into the otic labyrinth, after first removing a part of the latter. The following results were obtained:

In several cases where the fragment was "small," *i. e.*, consisted of iris only without retinal cells or with some very few of the latter, no lens-like structures were formed. In a number of instances, however, where the fragment was "larger," *i. e.*, containing more retinal cells, lentoids were formed (apparently from the iris), while in several other cases the transplanted fragment was transformed into a small eye with a lens, which latter Wachs considers as formed from the fragment of iris. However, in a number of instances (pp. 425, 426–428) where the transplanted fragment contained many retinal cells and was in good condition ("trotz guter Erhaltung") no lens-like structures were formed.

These results are, obviously, inconclusive. They certainly do not permit of Wachs's conclusion that the lentoids and lenses of the "positive" cases owe their origin to a stimulus from a secretion of the retinal cells (carried with the transplanted fragment of iris). For, why could not such effect of the retinal "secretion" be observed in the "negative" cases in spite of considerable retina? From these results I can read no definite, unmistakable, answer to the question which they were to answer.

For the following reasons, however, I should regard the query as a very pertinent one.

We know that the retina (Spemann, Lewis, Le Cron, Bell, Ekman, Werber and others) can furnish the stimulus for the formation of a lens from an epithelial derivative of the ectoderm (even epithelium of the mouth—Mencl, '08, and Werber, '16*c*) by contact with it. Besides being capable of furnishing the stimulus for this "lentogenic reaction" the retina (an ectodermal derivative) is according to Fischel's ('00, '02) and my (Werber, '16*c*) observations capable also of responding to such a stimulus—the lentoids of the retina. Whence does this stimulus issue in

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the case of the formation of the retinal lentoids? Considering the fact that the retinal cells can both furnish the "lentogenic" stimulus and respond to it, it would certainly seem not far fetched to assume that the stimulus for the formation of the lentoids by the cells of the retina issues from themselves. In this case the retinal cells react to their own "lentogenic enzyme." Both the conditions for furnishing the stimulus and the latent potency of responding to such a stimulus are known to be present in the retina. May not this double capacity underly also the formation of the lens from the iris?

To answer the latter question it would be necessary to find out whether the iris besides its known potency of forming a lens (Wolff, Müller, Fischel, Wachs, and others) is also capable of furnishing the "lentogenic stimulus," if in contact with epithelium (or some other ectodermal derivative). Unfortunately, however, the question cannot be answered definitely at the present time.

There exists only one experiment (by Wachs, *l. c.*, p. 430 and Figs. 46, 47, 48 and 49) which suggests this possibility, but the conditions in this experiment are not quite clear. A fragment of the iris was in this case (No. 39) transplanted into the cartilaginous capsule of the otic labyrinth. This fragment stimulated the formation of a lens (Fig. 49) from a strand of epithelium that had grown in from the edge of the wound. It is uncertain, however, whether in this case the "lentogenic" stimulus issued from the iris or perhaps from retinal cells contained in the transplanted fragment. For while on p. 429 Wachs states that this fragment was "ein Stück der oberen Iris" and that "nach 28 Tagen . . . war das Stück mit Goldpigment noch deutlich sichtbar," on p. 447 (in the summary) he refers to it as "ein Stück des Auges" and in the record (p. 423) this case (No. 39) is enumerated among those in which "ein Stück des oberen Augenteiles" was transplanted into the labyrinth. Wachs himself, however, does not state whether he assumes that in this case the regenerated epithelium formed the lens under the influence of retinal cells or of the iris.

There is no reason, however, why the latter possibility should be excluded. It is certainly not excluded by those experiments

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of Wachs in which a fragment of the eye cup containing both iris and retina (cf. Fig. 50) transplanted "dicht unter die Haut" has failed to stimulate lens-formation from the latter, but instead has so altered it that it gave the appearance "einer kleinen Cornea." For in these cases the failure to induce the lentogenic reaction might have to be attributed to the retinal as well to the iris-part of the transplanted fragment; it may, however, be due to the circumstance that the skin of the relatively old larvae was no longer capable of responding to a "lentogenic stimulus." The latter possibility may be implied from the (above-mentioned) case (pp. 237 and 241) in which owing to the operation an ingrowing strand of epithelium formed a lens under the influence of the transplanted fragment of the optic cup. For, as Wachs himself concludes, we are in that case (No. 39) dealing with "Abkömmlinge von Hautzellen, die, neugebildet und noch undifferenziert, offenbar die gleiche Fähigkeit der Linsenfaserbildung haben können, wie einst die junge Haut . . . " (*l. c.*, p. 430).

But while there is, as we see, no definitely known instance of lens-formation owing to a stimulus from the iris, there is, on the other hand, nothing that would contradict the assumption that the iris is capable of exerting such a stimulus on ectodermal derivatives. Moreover, the very fact that the other parts of the optic cup (retina and tapetum nigrum) can furnish the lentogenic stimulus would make it appear very probable that the iris which genetically is also a part of the optic cup (proper), is likewise possessed of the ability to induce the "lentogenic reaction."

Granting the correctness of this assumption (which eventually may be borne out by suitable experiments), we would in the case of the iris be confronted by the same conditions as in the case of the retina. It is known that the iris can form a lens, and if, as seems probable, it can also induce the formation of a lens from ectodermal epithelium, the assumption may be justified that in the formation of a lens from the pupillary edge of the iris, which is an ectodermal derivative, its cells respond to their own "lentogenic enzyme."

These conditions apparently obtaining in the formation of lentoids from the retina and very probably also in the "regeneration" (secondary formation) of the lens from the iris are in prin-

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ciple similar to the conditions obtaining in the primary (embryonic) formation of the lens, although the morphogenetic scheme of the latter cannot be fully applied to either the retinal lentoids or to the lens formed from the iris. The chief difference between the two modes of the morphogenesis of the lens is that while the origin of the "lentogenic stimulus" and the part responding to it are locally separate in the primary formation of the lens, they would—granting the correctness of our interpretation—coincide locally in the secondary formation of the lens from the iris or of a lentoid from the retina.

In order to fully understand the morphogenetic process underlying the "regeneration" of the lens from the iris (or of lentoids from the retina), it would also seem necessary to explain just how the "lentogenic stimulus," the latent capacity for which is probably possessed by the whole optic cup proper, is activated on the extraction of the primary lens. An answer to this query is, I believe, partly given by some of Fischel's ('02) experiments, which I shall review in the following.

The lens is so tightly enclosed by the iris that on its extraction slight injuries to the latter result. No matter how carefully the operation may be performed, slight, unnoticeable, lesions (abrasions?) of the cells of the pupillary edge of the iris may be unavoidable.[1] The significance of these, otherwise perhaps negligible, physical alterations of these cells of the iris resulting from the operation suggested itself to Fischel ('02, pp. 106–109 ff) from the observation of small lenses and lentoids formed not only by the pupillary edge but also from other parts of the iris. Being aware of accidental injuries to the eye cup and to the iris in some of his experiments he thought of a possible causal connection between the latter and such lenses or lentoids located in various parts of the iris. In order to test the correctness of his tentative interpretation he performed a number of experiments in which besides the exstirpation of the lens various parts of the iris were purposely injured. On microscopic examination of the results he was able to observe that small lenses or lentoids were formed

[1] According to E. Uhlenhuth (quoted from Loeb '16) who cultivated fragments of the iris by Harrison's explantation method, it is to these abrasions that the loss of the pigment by the cells of the iris is due. Owing to this injury of the cells the pigment granules are liberated into the lumen of the optic cup where, as Wolff ('95) has observed, they may be absorbed by leucocytes.

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from just such intentionally, and still discernibly distorted, parts of the iris. In several experiments Fischel observed besides the formation of a lens from the pupillary edge of the iris small double lenses, or twin (fused) lenses and in one case even three lenses in other parts of the latter. For the formation of these double (or multiple) structures Fischel, I think, correctly accounts by close proximity to each other of two (or more) places of injury.[1] Even the size of such lens-like structures he regards as dependent upon the extent (number of cells) of the injured area.

The lens formed by the pupillary edge of the iris after exstirpation of the primary lens is usually as large and as well differentiated as the latter. This, according to Fischel, is due to the circumstance that on extraction of the lens a large area comes under the stimulus of the injury, and also to the fact that the pupillary margin of the iris being an epithelial fold, it affords the best means for the formation of a lens-bud (the "Knoten"—Wolff '95), the first step in which is always a folding of the corresponding part of the epithelium.

Another apparent confirmation of this "Reizhypothese" was furnished by the lentoids of the retina. The latter were observed by Fischel ('02) in many experiments in which owing to the extraction of the lens the eye bulb sustained accidental injuries. The experimental test—intentional injury of the retina—again gave positive results. The "retinal lentoids" could be demonstrated to be due to transformation of thus injured cells or groups of cells of the retina into such lens-like structures. The fact that only the latter and not fully differentiated lenses of large size can be formed by the retina on mechanical stimulation is, according to Fischel, accounted for by the small area of such stimulation and by the difficulty of forming a large fold.

This ability of the retinal cells to become transformed into lens-like structures Fischel regarded already in 1902 as no more surprising than, (as demonstrated by his experiments), that of any part of the iris, or of the epithelium of the skin in normal

[1] In some experiments of Wachs (*l. c.*) in which a fragment of the iris was introduced into the posterior eye chamber of another animal whose lens has been extracted, both the implanted fragment and the animal's own iris formed a lens. In some of these cases where the fragment coalesced with the iris, two more or less fused lenses were observed at the point of coalescence, one formed by the foreign fragment and one by the iris.

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ontogeny. Already at that time he regarded it as a primary potency of all derivatives of the ectoderm. As the additional factor, however, necessary for this transformation of ectodermal derivatives he assumed an unknown stimulus which in the "regeneration" of the lens from the iris "*in jenen Alterationen zu suchen ist, welche das regenerierende Gewebe direkt durch den experimentellen Eingriff selbst erfährt* . . . von welchen die Zellen der Iris bei der Linsenextraktion betroffen werden" ('02, p. 106).

This unknown stimulus Fischel ('16) now regards as a chemical one from a secretion by the optic cup, or more specifically, from the retina which in conformance with Spemann and Wachs he also assumes. To the stimulus from this secretion he attributes the transformation of the supra-ocular epidermis into a lens in the embryo as well as the like transformation of the cells of any injured part of the optic cup (iris or retina).

While in the main I agree with Fischel by recognizing the necessity of an injury to that part of the iris or retina from which the secondary lens or lentoid arises, as proven, I am, as already stated, inclined to doubt the probability of a secretion by the eye cup as the additional factor involved in the stimulus for the formation of the secondary lens. Undoubtedly, with the present data at hand, it is difficult to deny that some chemical process is apparent in the morphogenesis of both the primary and the secondary lens. This process, however, may, as was pointed out in the preceding pages, be due to a substance (a "lentogenic enzyme") apparently contained by all parts of the eye cup already in the stage of the optic vesicle or even earlier. The activation, however, of this "enzyme" seems in the case of the "regeneration" of the lens to be in some way due to the injury of those cells of the iris which subsequently become transformed into lens fibers.

What would yet remain to be explained is the nature of the relation of the injury of the cells to the activation of their own "lentogenic enzyme." On this point, however, I should venture no opinion at the present time.

One apparent objection to our interpretation may yet be considered.

It is known from experiments by Wolff ('03) that if a part of

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the iris be removed by cutting (iridectomy) without removing the lens, the iris will regenerate but form no lens-like structures. Wachs (*l. c.*) attempted to account for this fact by the assumption of a secretion from the lens which would inhibit the formation of another lens. The results of some of his experiments, he believes, substantiate this conclusion. In these experiments the lens was removed and subsequently the lens of another animal of the same or a related species was introduced into the eye. In no case in which the foreign lens healed into the eye was a lens "regenerated." This result, however, permits of another interpretation, in which no resort is made to the improbable assumption of the "antisecretion" by the lens.

Indeed, Wachs's own ingenious experiments performed for the solution of this particular part of the lens-problem would seem to disprove rather than to prove the assumption of a secretion. For they show that the "regeneration" of a lens is inhibited only when the (smaller) lens implanted into the eye, heals into it, *i. e.*, comes into close contact with the iris ("so dass die verengerte Iris sich ihr ringsum dicht anschliesst . . . ," p. 404). If, however, such contact is not effected—"liegt jedoch die aus dem jüngeren Tiere implantierte kleinere Linse mehr oder weniger in der vorderen oder hinteren Kammer, so w'rd eine Regeneration eingeleitet."

Wachs has ascertained beyond doubt that the inhibition is in these cases due not only to the mechanical effect of the contact, but very apparently also to a chemical action. While, however, he strongly inclines to the belief that the chemical action is due to a secretion, I think it can easily be shown also in all other of his experiments by which the matter was tested, that this chemical action is conditioned by contact of the implanted lens with the iris.

These important experiments of Wachs, strengthened further by some interesting results of Fischel's ('02) experiments[1] would

[1] Fischel exstirpated the lens and replaced it by small spherical fragments of the potato tuber. Whenever this "imitation lens" was large enough to fit the pupilla, no "regeneration" of a lens from the latter took place. If, however, its diameter was smaller than that of the pupilla, partial "regeneration" of the lens or at least an "attempt" towards it was noted, complete "regeneration" being impossible owing to mechanical obstruction by the potato fragment. This experiment clearly demonstrates that the extraction of the lens eliminates not an indifferent body, but one with a specific, *inhibiting*, action.

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seem, however, to leave no doubt that the lens contains something, some substance that apparently inhibits the formation of another lens from the iris which would invariably occur in its absence.[1] This substance, however, again need not necessarily be secreted, in order to neutralize the action of the "lentogenic enzyme." Just what the nature of this substance may be, is, of course, altogether a matter of conjecture. It might perhaps be imagined as an "antibody" ("antigen") contained in the peripheral part (the epithelial cells) of the lens and the realization of its inhibiting action may be due to close contact of that part of the lens with the epithelium of the iris.

This "antibody" being, just like the lens, a product of the "lentogenic reaction," we may perhaps in the case of the vertebrate eye eventually have a striking example of some of the factors (synthetic enzyme action—Loeb, '16) concerned not only with development and growth, but also with the limitation of the latter[2] for the attainment of proper size relations, and with the maintenance of a chemical equilibrium indispensible for the undisturbed existence of a structure or an organ.[3]

[1] Fischel ('16) has recently furnished another interesting example of the apparent chemical action of the lens which he also considers as a "secretion." He transplanted in larvae (about 3 cm. large) of *Salamandra maculosa* the exstirpated lens under the skin of various parts of the head or trunk. As a result he observed that, while the lens underwent a gradual dedifferentiation and eventual absorption, the skin above the transplantate showed remarkable changes. The unicellular glands (the cells of Leydig) disappeared from the corresponding region of the skin, which, owing to the morphological changes, by its appearance suggested a similarity to "frühe Entwicklungsstadien des Hautepithels" (p. 37). Eventually this region of the skin became transparent and was very similar to a fully differentiated cornea.—Similar observations were, as mentioned above (p. 242), recorded also by Wachs (*l. c.*) on transplanting parts of a fully differentiated optic cup under the skin.

[2] The fact that the "regenerating" lens does not grow indefinitely (as it might, if it were a benign tumor—regarded as such at one time by Fischel, '00), but is limited in its growth to attain just the size to fit the pupilla, has recently occasioned J. Loeb ('16) to raise the question regarding the factors limiting growth. According to our assumption it might perhaps be imagined that every tissue (or structure) elaborates during its development a substance (an "antibody") which inhibits its growth beyond certain limits. In this case both growth and its automatic limitation would be conditioned by the same factors of development.

[3] Fischel ('16) concludes from his experiments that the already fully differentiated lens needs the chemical influence of the eye for its normal (undisturbed) existence.

OSBORN ZOÖLOGICAL LABORATORY,
YALE UNIVERSITY, October 4, 1917.

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SEX-DETERMINATION AND BIOLOGY OF A PARASITIC WASP, HADROBRACON BREVICORNIS (WESMAEL).

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The problem of sex-determination is nowhere of greater interest than in the Hymenoptera. The occurrence of parthenogenesis as well as sexual reproduction adds interest to this matter, especially in view of the fact that considerable variation in conditions obtains, not only between the different families, but even between the minor groups of the same family.

The present studies deal with an insect, *Hadrobracon brevicornis* (Wesmael), belonging to the family Braconidæ.

The insects vary greatly in coloration, ranging from near black though various intermediate patterns to near yellow. In the near-yellow individuals black pigment usually persists in the antennæ, in the compound eyes, about the ocelli, and in small markings near the base of the wings. No experiments have been made to test the cause of color variation except that it has been shown that it does not yield to selection. It seems probable, therefore, that it is due to some environmental influence. It shows a certain correlation with size, for the dwarfed specimens are usually very dark while the larger individuals are either very light or of intermediate color. There is no apparent correlation of color with sex.

In view of the fact that genetic differences with respect to color exist in the honey-bee, I had expected the yellow and the black variations of *Hadrobracon* to reproduce themselves, but this is not the case.

Fig. 1, *A*, shows a light colored male and Fig. 1, *B*, shows a rather dark female. Sexual dimorphism is evident in the longer antennæ of the male and in the presence of the ovipositor of the female. Variation occurs in the number of antennal joints and in the general body size. The larger specimens are three to four

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millimeters in length, but minute individuals occur as well. The latter are probably starved. They are fertile, nevertheless, and give rise, when bred, to wasps of normal size.

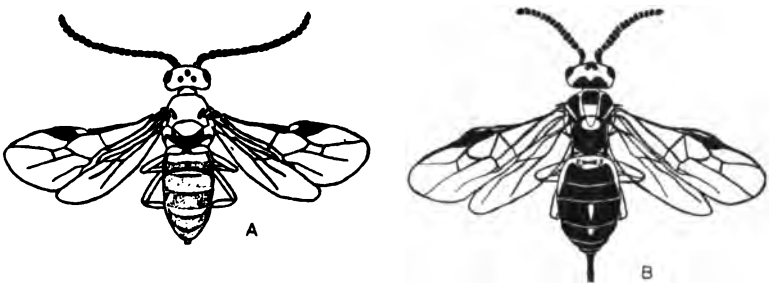

FIG. 1.

The insects mate readily as soon as they emerge from their white silken cocoons. The females deposit their eggs upon the bodies of full-grown caterpillars of the Mediterranean flour-moth, *Ephestia kuehniella* Zeller, and probably also upon other meal caterpillars. The caterpillars are stupefied by the sting of the wasps and remain quiet until the acephalous, apodous larvæ emerge and devour them. Several eggs are laid upon one caterpillar and as many as ten or a dozen wasps will sometimes develop. Usually the number is smaller. At a high temperature the length of a generation is ten days or less. The adult females may live as long as six weeks.

The work has been handicapped by lack of sufficient caterpillars at necessary times. As a consequence I have not been able to rear as many offspring as desired. The greatest number of progeny obtained from a single female was one hundred and twenty-eight, but I am convinced that many more could be secured if sufficient food were available. The technique of rearing and handling the moths is being developed and it is hoped that further work upon the wasps may be done as a result.

A protozoan parasite has been found in the caterpillars and in *Hadrobracon*. It has been eliminated by heat sterilization of the food and glassware. Its life history is being worked out by Dr. D. H. Wenrich. The small numbers in some of the families of *Hadrobracon* are due to the presence of this parasite.

My thanks are due to Dr. McClung for the facilities of the

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Table I.

Inbred Progenies of Mated Females.

Designation of Parents.	Offspring.		
	Generation.	Sex.	
		♂.	♀.
♀ no. 1	F_1	49	47
F_1a	F_2	34	56
F_1b	F_2	26	29
F_1c	F_2	31	48
F_1d	F_2	31	47
Total.....	F_2	122	180
F_2a from F_1a	F_3	6	17
F_2b " F_1a	F_3	3	9
F_2c " F_1a	F_3	8	10
F_2t " F_1a	F_3	2	10
Total.....	F_3 from F_1a	19	46
F_2d from F_1b	F_3	10	9
F_2q " F_1b	F_3	2	8
F_2p " F_1b	F_3	11	8
F_2o " F_1b	F_3	21	13
F_2n " F_1b	F_3	5	13
F_2k " F_1b	F_3	15	18
Total.....	F_3 from F_1b	64	69
F_2e from F_1c	F_3	4	12
F_2l " F_1c	F_3	25	18
Total.....	F_3 from F_1c	29	30
F_2i from F_1d	F_3	2	6
F_2j " F_1d	F_3	1	5
F_2m " F_1d	F_3	13	13
Total.....	F_3 from F_1d	16	24
Total.....	F_3	128	179
F_3l from F_2a	F_4	5	23
F_32 " F_2a	F_4	17	18
F_33 " F_2a	F_4	18	19
F_34 " F_2a	F_4	11	27
Total.....	F_4 from F_2a	51	87
F_36 from F_2q	F_4	15	28
F_37 " F_2q	F_4	11	31
F_38 " F_2q	F_4	10	23
F_310 " F_2q	F_4	7	10
Total.....	F_4 from F_2q	43	92
F_3 12 ♀ from F_2t	F_4	21	39
Total.....	F_4	115	218

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TABLE I.—*Continued.*

Designation of Parents.	Offspring.		
	Generation.	Sex.	
		♂.	♀.
F_4a	F_5	4	6
F_4b	F_5	8	22
F_4c	F_5	9	12
F_4g	F_5	4	3
F_4p ♀ from $F_3 6$	F_5	19	19
F_4w ♀ " $F_3 6$	F_5	12	12
F_4y ♀ " $F_3 8$	F_5	26	25
$F_4 14$	F_5	32	48
$F_4 15$	F_5	7	19
Total.....	F_5	121	166
F_5a	F_6	14	9
F_5g	F_6	2	9
F_5m	F_6	5	6
Total.....	F_6	21	24
F_6g2	F_7	2	3
F_6g3	F_7	8	7
F_6k	F_7	2	4
F_6h	F_7	1	1
Total.....	F_7	13	15
F_7b	F_8	1	1
F_7c	F_8	8	9
F_7d	F_8	2	3
F_7e	F_8	0	1
Total.....	F_8	11	14
F_8a	F_9	4	2
F_8d	F_9	9	1
Total.....	F_9	13	3
	Total from 51 mated females	593 ♂♂	846 ♀♀

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In November, 1916, a female wasp (♀ No. 1) was found in cultures of the Mediterranean flour-moth at the Zoölogical Laboratory. A little later some cocoons of the wasp were found and these were isolated in vials. Both males and females emerged from the cocoons. The females were not mated but produced parthenogenetically families consisting entirely of males.

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The first female (♀ No. 1), produced forty-nine males and forty-seven females. As later results show she had in all probability mated. Table I. shows the progeny of mated females descended from ♀ No. 1. No variation of sex-ratio correlated with age of the mother or lapse of time since mating could be detected.

The first column of Table I. gives the designation of the parents. The various matings of a given generation are designated by the letters of the alphabet, as F_3a, F_3b, etc. In case there are more than twenty-six matings in any generation, the succeeding matings are numbered, as $F_3$1, $F_3$2, etc.

Omission of letters or numbers denotes that the parents in question are elsewhere summarized or else produced no offspring.

The second column denotes the generation of the offspring and the third and fourth columns denote the males and females respectively.

The points of interest to be noted in Table I. are as follows:

Each of the fifty-one females produced females, and all but F_7e, which produced a single female only, produced males.

Considerable variation in sex ratio obtains in different fraternities, usually with excess or equality of females.

An attempt to correlate sex ratio in the maternal fraternities with that of the progeny resulted as follows:

Matings of progeny of	F_1a	(34 ♂♂ : 56 ♀♀)	gave	(19 ♂♂ : 46 ♀♀)
"	F_1c	(31 ♂♂ : 48 ♀♀)	"	(29 ♂♂ : 30 ♀♀)
"	F_1d	(31 ♂♂ : 47 ♀♀)	"	(16 ♂♂ : 24 ♀♀)
"	F_3a	(6 ♂♂ : 17 ♀♀)	"	(51 ♂♂ : 87 ♀♀)
"	F_3q	(2 ♂♂ : 8 ♀♀)	"	(43 ♂♂ : 92 ♀♀)
"	F_3t	(2 ♂♂ : 10 ♀♀)	"	(21 ♂♂ : 39 ♀♀)
"	$F_3$6	(15 ♂♂ : 28 ♀♀)	"	(31 ♂♂ : 31 ♀♀)
"	$F_3$8	(10 ♂♂ : 23 ♀♀)	"	(26 ♂♂ : 25 ♀♀)

Fraternities with excess females (131 ♂♂ : 237 ♀♀) gave 236 ♂♂ : 374 ♀♀ = 1 : 1.6.

Matings of progeny of	♀ no. 1	(49 ♂♂ : 47 ♀♀)	gave	122 ♂♂ : 180 ♀♀
"	F_1b	(26 ♂♂ : 29 ♀♀)	"	64 ♂♂ : 69 ♀♀

Fraternities with equality of sexes (75 ♂♂ : 76 ♀♀) gave 186 ♂♂ : 249 ♀♀ = 1 : 1.3.

It is seen that a higher female ratio is obtained in the progeny of those fraternities in which the females were in excess of the

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males than in the progeny of those fraternities in which the sexes were equal, but although this condition obtains the numbers are too small and the exceptions too numerous to be conclusive. Thus the progeny of F_1c, F_36 and F_38 had excess of females but produced equality of sexes, while the progeny of ♀ No. 1 had equality of sexes but produced excess of females.

As mentioned earlier in the paper, virgin females gave only male offspring.

Twenty-six virgin females were isolated and produced 757 males. The fraternities consisted of 4 to 84 individuals. Eight females that were with males produced 146 males and no females. It is probable that they did not mate.

TABLE II.

OFFSPRING OF FEMALES, BEFORE AND AFTER MATING.

Designation of Parents.	Offspring.		
	Before Mating, ♂♂.	After Mating.	
		♂♂.	♀♀.
		to other than their own sons	
v ♀ 2	106	13	9
$F_3$11	7	5	2
F_3c	20	1	6
		to their own sons	
F_3f	7	5	7
F_4i	59	51	0
F_4n	16	11	27
F_4o	50	11	11
$F_4$1	30	0	2
F_5j	41	42	6
F_6a	40	2	2
Total from ten ♀♀	376 ♂♂	90 ♂♂ (excluding F_4i)	72 ♀♀

Total from thirty-six virgin females—1,133 ♂♂.

Total from sixty-one mated females (excluding those that were set with males but produced only males)—683 ♂♂, 918 ♀♀.

Table II. shows the progeny of ten females that were mated late in life after they had produced several males. Seven of these were mated to their own parthenogenetically produced sons. In every case only males were produced before the mother mated and nine of the mothers produced daughters after mating. Virgin female F_4i produced fifty-one males after being introduced to a male. It is probable then that she did not mate.

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The excess of males (90 : 72) in the progeny of females mated late in life is probably due to the fact that they did not mate after being set with a male until after they had laid a few eggs.

Summarizing then we have sixty-one females set with males that produced both males and females (683 : 918), nine that produced only males (197) and thirty-six virgin females that produced only males (1133). *Not a single female has been produced from a virgin female.*

The conclusion might be drawn then that in *Hadrobracon brevicornis* fertilized eggs produce females and unfertilized eggs produce males. If this is comparable with conditions in the honey-bee and the hornet, it must be supposed that the male is a haplont; the female, a diplont. An alternative possibility would be that males are diplonts, in which case they might be formed either from unreduced eggs or from reduced eggs that have been fertilized by male-determining spermatozoa.

Cytological work is now in progress that confirms the theory that the male is haplont. The first spermatocyte division is abortive as in the honey-bee. Details will be published later.

Other work in regard to sex-determination in the Braconidæ is that of F. M. Webster (1909) and S. J. Hunter (1909) on *Lysiphlebus tritici*, belonging to the subfamily Aphidiinæ. The results of these investigations have been reviewed and discussed by A. F. Shull (1910). Both males and females were produced from mated females. Virgin females usually produced males but occasionally a few females.

The work done at the United States Parasite Laboratory at Melrose Highlands, Mass., indicates that males are usually produced from virgin females of Braconids and Ichneumonids, but Hemiteles, an Ichneumonid hyperparasite, produces females parthenogenetically as well.

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STUDIES IN THE SEX-RATIO IN MAN.

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The numerical proportion of the sexes has always been a subject of great interest. The question whether they occur with equal frequency in man bears a close relation to the problem of sex-determination and comparative sex mortality. While the original sex-ratio is conditioned upon sex-determination, mortality may in the course of time change it. It will be shown that sex-ratio is not constant but varies in the different periods of life and under manifold influences. Most of the information at hand in regard to sex-ratio is derived from statistics of new-born and adults, but as regards intra-uterine life as far back as conception, our knowledge on the subject is limited to a few conflicting estimates. One of the purposes of this paper is to elucidate as far as possible this last problem. Inasmuch as the supposition that sex is determined before or at the time of fertilization is generally accepted, one may speak of a sex-ratio of conceptions, which may also be called original, physiological, or primary sex-ratio. That at birth, therefore, should be termed secondary and finally the sex-ratio of adults, tertiary.

TERTIARY SEX-RATIO.

The consideration of sex-ratio in adults will be limited to general remarks. With only a few exceptions European countries have a greater proportion of women than men. According to Rauber ('00) the sex-ratio[1] including all ages, for all Europe, is 97.8; for Asia 104.6; for Africa 103.3; for America 102.2, and for Australasia 114.3. According to Brooks ('87) the Australasian colonies had in 1881 a sex-ratio of 143.72 for the aborigines, and one of 118.64 for the population of foreign descent. However

[1] The most common method in use for representing the average sex-ratio is to determine a number which indicates the proportion of males to every hundred females. Unless otherwise stated, this is the method herein used. The sex-ratio is also frequently called *masculinity*.

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imperfect these computations may be, they nevertheless show that Europe, with its excess of females, assumes a unique position. The following statistics, taken from one of Rauber's tables, represent the number of females for every 1,000 males:

Age in Years.	Austria, 1869.	England and Wales, 1871.	France, 1872.	Germany, 1875.
25–30	1,087	1,111	1,017	1,059
30–40	1,074	1,092	991	1,046
40–50	1,089	1,081	996	1,052
50–60	1,033	1,074	1,008	1,081
60–70	1,022	1,128	1,033	1,114
80–90	1,052	1,376	1,359	1,240

With advancing age the excess of females increases. This is still more clearly shown in a table by Prinzing ('05) taken from the census of 1900 in Germany:

Number of females for every 1,000 males:

0– 5 years	993	40–45 years	1,045
5–10 "	998	45–50 "	1,067
10–15 "	995	50–55 "	1,121
15–20 "	995	55–60 "	1,140
20–25 "	1,008	60–70 "	1,189
25–30 "	1,008	70–80 "	1,259
30–35 "	1,014	80–90 "	1,338
35–40 "	1,020	90– "	1,751

Inasmuch as in Europe sex-ratio at birth favors males, its subsequent decrease must be the result of a greater mortality among the latter, and in some countries, also, of a greater emigration of males. The unequal mortality is shown in a table by Ploss ('87). For every 100 females there occur the following numbers of male deaths:

Sweden	104	Spain	107
Russia (Europe)	105	Austria	108
Holland	105	Switzerland	108
Italy	106	Germany	109
England	107	Greece	111
France	107	Rumania	116

The sex-ratio of mortality may differ under various circumstances; for example, between the ages of 25 and 40 years according to Knöpfel ('07) in rural districts it is below, while in the urban population it is above 100.

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Secondary Sex-Ratio.

The sex-ratio of the living-born is, on a rough average for all European countries, 105–106; but this number may vary markedly even in comprehensive statistics. Manifold explanations for these variations have been offered and these will be considered briefly farther on. In the individual countries small differences in the secondary sex-ratio may exist, as shown in the following examples taken from a table by Ploss:

Russian Poland	101	Denmark	105
England and Ireland	104	European Russia	105
France	105	Italy	106
Germany	105	Austria	106
Switzerland	105	Massachusetts	106
Belgium	105	Spain	107
Holland	105	Connecticut	110
Sweden	105		

Concerning countries outside of Europe there is little information. Newcomb ('04) states that in Japan the excess of males in more than a million births was practically the same as in European countries. According to the same author, it seems probable that among the negroes of the United States there is a slight excess of female births.

As a result of the unequal mortality of the sexes the secondary sex-ratio becomes reversed early in life. Kroon ('17) states that in Holland the sex-ratio of mortality during the first year of life is 119; that is, the mortality among male infants is one fifth again as great as among females. He also states that during the first two months of life this ratio reaches even 139. Analogous numbers for the first year of life are given by Prinzing ('06):

Italy	111	England	121
Rumania	115	Sweden	121
France	119	Denmark	121
Austria	119	Norway	123
Switzerland	120		

Kroon's statistics show that this high sex-ratio of mortality of the first year soon decreases, reaching its minimum—approximately 80—between the fourteenth and fifteenth year. According to Prinzing ('05) the mortality from the ages of 5 to 20 years is greater in females, and indeed in the latter part of this

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period it is the result chiefly of tuberculosis, for which disease the common occurrence of anæmic and chlorotic conditions at the time of puberty furnishes an excellent soil. After this age the sex-ratio of mortality increases rapidly and results in the reversion of an excess of males to an excess of females.

Primary Sex-Ratio.

The sex-ratio of conceptions cannot be determined directly; however there is an indirect method of solving the problem of the original sex-ratio by means of computing the mortality of embryos and fœtuses. Only in case the sex-ratio of those dying in utero is equal to the secondary sex-ratio, will the primary be equal to the secondary. If male abortions were absolutely the same as female, then the primary sex-ratio would be smaller than the secondary. If the sex-ratio of mortality during pregnancy exceeds the sex-ratio of the livingborn, then the sex-ratio of conceptions will of necessity be greater than the secondary and indeed all the more so as the total mortality becomes relatively greater. It is necessary, therefore, to consider two factors in order to deduce the primary from the secondary sex-ratio. The following schematic representation will illustrate this:

1. How many male abortions and stillbirths occur to every 100 female abortions and stillbirths?

2. How many abortions both male and female, occur to every 100 living born?

100 living born with sex-ratio $105.5 + a$ stillbirths and abortions with sex-ratio b.

$100 + a$ conceptions with sex-ratio x.

This scheme becomes complicated by differences in sex-ratio of mortality and in the relative rate of mortality in the various periods of pregnancy. The relative number of stillbirths differs to some extent according to the various authors. This is in part explained by the fact that the statistics are taken from various countries. Rauber states that the stillbirths amount to 4 per cent. of the annual 1,800,000 births in Germany. According to Carlberg ('86), the proportion of stillbirths to the total number of births in Livland lies between 2.58 and 2.90 per cent., while the percentage for Western Europe lies between 4 and 4.5 per

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cent. According to Prinzing ('07), reports show that from 1891 to 1900 in every hundred births there were the following proportions of stillbirths:

Austria	2.9	Holland	4.3
Switzerland	3.6	Belgium	4.5
Italy	3.9	France	4.6

These figures are somewhat increased when expressed in percentages of livingborn. Computing from Auerbach's ('12) statistics of over 100,000 births in Budapest, the stillbirths amounted to 3.3 per cent. of the livingborn. Bucura ('05) found that among 40,169 births in the Clinic Chrobak in Vienna 5.8 per cent. were stillborn; Le Maire ('06) found 5.7 per cent. among 40,339 births in Copenhagen. Both of these figures are too high, inasmuch as these authors did not use the term *stillborn* in the usual sense, a small number of abortions being included. Bernoulli accepts 4 to 5 per cent. stillbirths to the total number of births.

The relative number of abortions is extremely difficult to determine, inasmuch as everywhere large numbers, especially of the earlier months, remain unknown. Williams ('17) expresses himself on this point as follows: "A conservative estimate would indicate that about every fifth or sixth pregnancy in private practice ends in abortion, and the percentage would be increased considerably were the very early cases taken into account, in which there is profuse loss of blood following the retardation of the menstrual period for a few weeks." Other obstetricians give different estimates. Franz ('98) found 15.4 per cent. of pregnancies ending in abortion, Malins ('03) 19.23 per cent. Taussig ('10) estimates that one abortion occurs to every 2.3 labors, Pearson ('97) to every 2.5 labors. Auerbach reports that according to estimates for Berlin, abortions amount to one-sixth to one-tenth of the number of livingborn. According to the same author, there were in Budapest in 1901–05, 111,139 living born, and in the years from 1903–05, 7,702 abortions. Assuming an approximately equal number of livingborn for each year, the livingborn between 1903 and 1905 would amount to 66,678, of which number there would be 11.55 per cent. abortions. This percentage is doubtless too small;

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Auerbach himself assumes that many abortions of the earlier months are concealed. The same author found that of his material 3,635 abortions, or almost half, fell within the first three months of pregnancy, and it is for this very period that the statistics are incomplete; therefore in reality much more than half the number of abortions belong to the first three months. According to Franz 42.6 per cent. and according to Dührssen even 59 per cent. of abortions occur in the third month alone.

The sex-ratio of stillborn is much higher than that of living-born. A table taken from Morgan ('13) gives the following sex-ratios of stillborn for European countries:

Norway	124.6	Denmark	132.0
Holland	127.1	Belgium	132.0
Germany	128.3	Austria	132.1
Hungary	130.0	Switzerland	135.0
Italy	131.1	Sweden	135.0

Such high sex-ratios of stillborn as that of Walter (260) or that of Tschuprow (400) are probably based upon relatively limited material and do not represent true ratios.

Auerbach gives detailed information concerning the sex-ratio of abortions. This author's material is distributed among the different months as follows:

7th	month	402 ♂	348 ♀	sex-ratio 116
6th	"	506 ♂	437 ♀	" " 116
5th	"	645 ♂	396 ♀	" " 163
4th	"	928 ♂	405 ♀	" " 229
4th–7th	"	2,481 ♂	1,586 ♀	" " 156.4

For the first three months of pregnancy he assumes that the sex-ratio of abortions is at least equal to that of the fourth month, namely 229. It seems to him more probable, however, that it increases in constant proportion; therefore he estimates 322 for the third month and 452 for the second month. According to Carvallo ('12) the sex-ratio of dead embryos up to the fourth month is 250; this figure he calculated from the statistics of Paris in 1908. Körösy ('98) found the sex-ratio of 3,781 abortions to be 152.4. Pinard and Magnan ('13) report on 1,229 abortions, the age of which is not stated. This material showed a sex-ratio of

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only 101.1. Rust ('02) also found the sex-ratio of 454 abortions from the first six months very low, namely 101.8. It is apparent how greatly these figures vary. A new contribution towards the knowledge of sex-ratio of abortions, even if based upon a relatively small amount of material is, therefore, not valueless, especially inasmuch as great care has been taken to determine age and sex. The material for these statistics comprises almost 600 fœtuses from the collection of the embryological department of the Carnegie Institution. Age classification was based upon the sitting height (Keibel and Mall '10). Rauber published the sex of 57 fœtuses with their sitting height; these have been made use of with the author's material. Normal and pathological fœtuses, for the most part white, were used in this study. In a limited number of cases no parental history was available; however, it is very probable that the majority of these also were white. Among the few specimens of races other than white, there is a preponderance of negro. The total material from three to ten months comprises 332 males and 315 females, showing a sex-ratio of 105.4. Rauber's material alone gives a sex-ratio of 159. The sex-ratio of the definitely white fœtuses amounts to 118.7. In the various months it is as follows:

Month.	Total.	White.
3d	123.7	108.3
4th	110.5	109.4
5th	108.1	163.4
6th	58.8	76.0

The material upon which are based the sex-ratios from the seventh to the tenth month is too limited to be of much value when represented for each month. The exceptional sex-ratio of the sixth month must be explained as a chance occurrence. The same may be true for the sex-ratio (78.9) of all the pathological fœtuses. According to the latter, female abortions are more frequently pathological, a finding which is difficult to correlate with the greater mortality of male fœtuses. The greatest deviations in the ratios obtained by Auerbach and Carvallo on the one hand, and by the author on the other, occur in the third and fourth months, for which Auerbach found the ratios to be 322 and 229, and Carvallo, 250. The author's corresponding figures are as

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low as 124 and 110. The great excess of male abortions in the early months of pregnancy, as found by Auerbach and Carvallo, may find its explanation in the fact that in the statistics used by them the sex of the fœtuses was determined by various individuals, who had not the necessary specialized knowledge. Early in the differentiation of the external genitalia only the expert can state the sex with certainty. At this time, and even later, the inexperienced, misled by the size of the clitoris as well as by other factors, may erroneously determine the fœtus as male. Fewer errors would be made if only those cases definitely male were reported as such, and all the doubtful cases were designated as female. Even granted that larger statistics might raise the sex-ratio, the latter would never reach the high figures stated by Auerbach and Carvallo and assumed by others. Just as sex-ratio of mortality following birth varies according to age, so is it found to be true for pregnancy. Nothing is known in regard to the sex-ratio during the first two months of pregnancy; however that of the third month might be used hypothetically for this period. The mortality of male fœtuses in the third month, which is about one fourth again as large as for females, is followed by a mortality in the fourth to the seventh months which is approximately relatively equal, for both sexes. During the eighth to tenth month this relation again shows a higher mortality for the males. The author's material from the fourth to the seventh month shows a sex-ratio of 101.1. However, on account of the great variability in the individual months, it is quite probable that of the male and female fœtuses that survive the third month, the number that perish during the following period up to the seventh month is relatively, not absolutely, equal.

If one proceeds to make use of the above citations and figures in computing the primary sex-ratio, rough and approximately average values must first be established. The following appear to be most probable:

For each 100 living born			with	sex	ratio	105.5
8th–10th month	4 still born		"	"	"	130
4th–7th "	9 abortions		"	"	"	106.3
0 3rd "	14 abortions		"	"	"	125
	127 conceptions		"	"	"	x

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From this, x or the primary sex-ratio is found to be *108.47*. The relative number of conceptions was estimated by Rauber to be 100 to 76 living born or calculated as above 131.6 to 100 living born. This number is somewhat larger than that obtained by the author, namely 127. The sex-ratio of conceptions was estimated by Bernoulli as 108.2. Slightly higher (108.7) is the ratio computed by Jendrassik ('11) from statistics collected by Bodio. Both these figures are strikingly similar to that of the author, namely 108.47. Lenhossék ('03) estimates the primary sex-ratio as 111; Auerbach as 116.4 but the latter believes that it would reach at least 125 if corrections were made.

Even if these approximate averages, which become constant only when based upon extensive material, must be accepted *cum grano salis*, it may nevertheless be stated with certainty that more males (probably not more than 10 per cent.) are conceived, and that at certain periods of pregnancy the relative mortality of males exceeds that of females by as much as one fourth.

Determination of and Changes in the Primary Sex-Ratio.

Now arises the question as to what determines this unequal distribution of sexes at conception. Its discussion dates back into antiquity, Hippocrates and Galen asserting that males originated from the right testis or the right ovary, females from the left. It has been proved that this theory is untenable; however, the idea of the possibility of two distinct varieties of spermatozoa was again revived in more recent times. Wilson ('05) distinguishes male-producing and female-producing spermatozoa. This might lead to an unequal distribution of sexes at conception. Morgan suggests that it may be due to a difference in the rate of travel of the two types of sperm, or that a disease process, or a factor such as alcoholism, might affect one type to a greater degree than the other. Hertwig ('12) attributes sex-determination to the ovum or the degree of its maturation, an advanced stage of maturation producing males. In this way he attempts to explain the difference in sex-ratio according to social class. Thury ('63) had proposed the idea that ova which are fertilized late may produce more males. Thus he explained the high sex-ratio among Jews, who, on religious grounds refrain from inter-

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course for seven days following menstruation. Lorenz ('98), Lenhossék and Orschansky ('03) are of the opinion that sex is subject to hereditary influences, inasmuch as they found families in which males predominated and those in which females appeared in excessive numbers. Newcomb, Woods ('06) and Heron ('06) deny this and show that inheritance plays no part in the sex-ratio. Numerous authors attribute its variations to the absolute and relative ages of the parents. According to Rosenfeld ('00) there is a decided preponderance of male children born to young and old fathers, as compared with those of middle age. Francke, from the statistics of Norway, found this to be true in respect to young fathers, but reached an opposite conclusion as regards old ones. Dumont ('94) found for Paris a sex-ratio of 101.9 when the fathers were from 18 to 25 years; 104.2 when the fathers were between 26 and 50 years and 97.5 when the fathers were over 51 years. According to E. Bidder ('78), the sex-ratio of births by mothers under 18 years and over 40 years is especially high. Sadler ('30) stated that the relative ages of the parents determines the sex-ratio; the latter is 86.5 when the father is younger than the mother, 94.8 when both are of equal age, and reaches 163.2 when the father's age exceeds the mother's by 16 or more years. Kollmann ('90) obtained an opposite result. He therefore draws the conclusion, based upon extensive material, that the sex-ratio is high when the father is younger, and low when he is older than the mother. At the same time he opposes the view that the absolute age of the mother has any influence whatever upon the sex-ratio. Stieda, on the basis of his investigations, reaches the conclusion that any influence of the absolute ages of the parents is out of the question, as he noted the highest sex-ratio when the parents were of equal age. Numerous other authors have occupied themselves with the question of parental age as an influence upon sex-ratio, but only two additional ones will be mentioned, Boudin ('62) and Stadler ('78). The conflicting views which have been presented suffice to show that nothing definite is known concerning a correlation between the age of parents and sex-determination; in fact such a correlation is hardly to be expected. Pearl ('08) in a very careful study demonstrated that there are more males produced when the

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parents are of different racial stocks. The well-known assertion that sex-ratio rises after wars, has evoked various attempts at explanation. The following few examples are given: Ploss ('58, '61) ascribes it to malnutrition of the mothers. Berner ('83) believes it to be due to the diminished concurrence which follows wars and which brings about an increased prosperity. Düsing sees the cause in the increased sexual demands upon the male, which also is said to increase the sex-ratio in polygamy. According to Newcomb, following the Civil War no increase in sex-ratio was observed. In the cases where a difference was confirmed it was so slight[1] as not to exceed the normal variations as shown by Lehr ('89), Carlberg and others, and is to be considered as such. Variations of sex-ratio have been determined not only for individual years and for groups of years, but also for the seasons. According to Goehlert ('89) in autumn and winter relatively few conceptions take place, but a higher percentage of these are male.

Changes in the Secondary Sex-Ratio.

The primary sex-ratio, as shown above, becomes transformed by an unequal intrauterine mortality of the two sexes into a different secondary sex-ratio. The greater mortality of males during certain periods of prenatal life is explained by Carvallo as follows: "les garçons sont plus fragiles." Auerbach also considers the male fœtus less resistant. Grassl ('12) finds an explanation for the difference in the viability of the germ plasma. Jendrassik speaks of hereditary reduction of vitality among the excess of males. Rauber explains the greater mortality of males by the greater demands of the larger fœtuses upon the mother, the latter not always being able to meet them; the production of a female does not require as much from the mother. Lillie ('17) offers the suggestion that the greater mortality among male fœtuses is a result of disturbance of the equilibrium that protects the male from the sex-hormones of the mother. It seems probable that this is the case, especially in the first part of pregnancy.

[1] For example Henneberg's ('97) statistics show that in Holstein between 1835 and 1845 the sex-ratio was 105.76, after the period of war from 1846 to 1853 it was 106.67.

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The excess of male stillbirths is ascribed by most authors to the more difficult labor attendant upon the greater size of the male, especially the circumference of the head. In regard to this question Dutton ('10) is of the opinion that at the time of birth the bones of the male skull are as a rule more firmly ossified than those of the female. In this connection he states also that with the advance of civilization the pelvic development in women is not proportionate to the cephalic development which is taking place in infants. This perhaps explains the fact shown by Bluhm ('12) that the relative number of induced premature births is on the increase. That labor in cases of male children more often demands artificial aid from the obstetrician is shown by Prinzing, according to whom 6.18 per cent. of male births in Württemberg called for operative measures, as compared with 4.67 per cent. in the births of female children. The process of labor itself is the cause of only a small percentage of stillbirths, the majority dying before labor sets in.[1] Therefore, the greater size of the male cannot in itself be held responsible for the high sex-ratio of stillborn. According to Treichler ('95) 29.6 per cent., and according to Prinzing ('07) 32.6 per cent. of all stillbirths are premature, and in the sex-ratio of these size plays but little part.

Frequently sex-ratio has been studied in relation to the pelvic diameters of the mother. The results are somewhat conflicting. Hoffmann ('87), Dohrn ('88) and Orschansky ('94) may be mentioned, according to whom the sex-ratio in children of mothers with narrow pelves is small; in contrast to this, Linden ('86) states it to be 133 in 360 births in which the mothers had narrow pelves. In case the size of the pelvis really has an influence, this can be exerted only upon the secondary sex-ratio in the way of elimination. In the same manner it seems evident that many of the factors which apparently affect the sex-ratio do not change it at conception, that is, they do not have any sex-determining effect, but by their influence upon intrauterine mortality they change only the sex-ratio of births. The well-known fact that the secondary sex-ratio among Jews is relatively high is explained by Düsing on the ground of incest, blood marriages being of

[1] According to Ladame ('04) those dying during labor amounted to 36.4 per cent. of all the stillborn in Switzerland in 1900.

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frequent occurrence in that race. Schultze ('03), on the other hand, has demonstrated that inbreeding has no effect upon sex-determination. The relative infrequency of abortions and stillbirths among Jews, as has been shown, for instance, by Auerbach for Budapest, explains very simply the fact that the secondary sex-ratio among Jews is higher and therefore closer to the primary. Punnett ('03) and others have shown that the births among classes of lower social status present a lower sex-ratio than those of the rich. The explanation lies again in the fact that the greater frequency of abortions among women of the working classes who can spare themselves less during pregnancy and in whom pregnancies occur in more rapid succession[1] results in a greater reduction in the sex-ratio, which was originally equal in the two classes. In addition, this greater reduction of the primary sex-ratio in the poorer classes is due to the higher percentage of stillbirths; according to Conrad in Halle among laborers it was 5 per cent., while among upper classes it was only 2.1 per cent.; and according to Verrijn Stuart ('01) in Holland, among the poor 3.16 per cent. and among the rich 2.50 per cent. of all births. A further example illustrating how the primary sex-ratio was erroneously thought to be influenced is shown in its difference between legitimate and illegitimate children. Heape ('09) states that the sex-ratio of legitimate births among the white population of Cuba is 109.0, stillbirths included; that of the illegitimate only 105.95; there is even a greater difference among negroes, the sex-ratio being 97.91 for illegitimate children and 107.73 for legitimate ones. Heape immediately draws the conclusion that illegitimate unions result more often in the conception of females than do legitimate unions. According to Düsing the sex-ratio of legitimate births in Prussia, between the years of 1875 and 1887, was 106.37, that of illegitimate only 105.54; the stillborn in legitimate unions amounted to 3.91 per cent.; in illegitimate 5.32 per cent. A corresponding difference was demonstrated by Bertillon ('96) in the frequency of legitimate and illegitimate abortions. The greater mortality of illegitimate fœtuses reduced the sex-ratio to a greater degree. The rule that the sex-ratio is greater in

[1] According to Düsing ('84) the longer the intervals between births, the greater is the sex-ratio.

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legitimate than in illegitimate births is not, however, without exception. Srdinko ('07) found that the sex-ratio of legitimate births in Austria was lower than that of the illegitimate, and explains this by the fact that the illegitimate are for the most part Jewish, in which race abortions are less frequent. According to a number of authors, the sex-ratio of first born is greater than that of subsequent births.[1] It is especially high in older primiparæ, as shown by Ahlfeld ('72 and '76), Janke ('88) and Bidder ('93). Lewis ('06) reports that in Scotland the sex-ratio of first born was 105.4 and that of subsequent births 104.8. That this at least in part is also due to different intrauterine mortality may be supported by the following citations. According to Franz, abortions are more than twice as frequent in multiparæ than in primiparæ. Moreover, the first-born children are appreciably smaller than subsequent ones, as demonstrated by Schaetzel ('93), a condition which would suggest a lower rate of mortality before birth as a result of the relatively fewer demands made upon the mother (Rauber). Hansen ('13) states that in Denmark the first-born weigh on an average 3,457 grams; the second-born 3,607 grams, third-born 3,698 grams, the difference between the first and second being much greater than between the subsequent ones. Stillbirths, however, according to Duncan and Duke ('17) are more frequent among first-born than among second- and third-born, in spite of their smaller size; only in the case of children from the sixth pregnancy dies the percentage of still born exceed that of first-born. However, inasmuch as many more abortions than stillbirths occur, comparatively little importance can be attached to this.

Besides the above mentioned causes for the variations found in sex-ratio, many others have been discussed in the literature. Only a few of these factors actually exert any influence upon the sex-ratio of conceptions. The changes have all been found in the secondary sex-ratio and the probability is great that the factors causing them play not a sex-determining but a sex-eliminating rôle. This is true chiefly in regard to changes resulting from locality, such as rural and urban districts, from age, nutri-

[1] A table demonstrating this may be consulted in Newcomb's article.

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tion and health[1] of the mother. To what extent race determines the sex-ratio of conceptions cannot as yet be stated; however it is not improbable that differences exist in various races.

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[1] Schenk ('98) asserts that diabetic mothers bear more female than male children.

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BIOLOGICAL BULLETIN

SPERMATOGENESIS OF THE PACIFIC COAST EDIBLE CRAB, *CANCER MAGISTER* DANA.

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1. INTRODUCTORY REMARKS.

The Decapoda have always been of absorbing interest to the cytologist and considerable has been written concerning their germ cells. In a previous publication ('14), I have given a review of the important literature on the subject, and by referring to this article it becomes apparent that practically all the investigations have been confined to the male reproductive elements of the Macrura and Anomura. Virtually nothing has been done with the Brachyura; in fact there isn't a single complete spermatogenesis of a crab known. Carnoy ('85) observed and pictured a few of the early proliferation stages in the sper-

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matogenesis of *Carcinus menas*, while Binford ('13) hastily described the spermatogenesis of the Atlantic coast edible crab, *Menippe mercenaria*. None of these investigators, however, have given details regarding the interesting processes of synapsis, reduction, chromosome numbers and the like.

During the past few years the writer has been gathering testicular material of numerous crabs which occur along Puget Sound, for the purpose of studying their spermatogenesis, and in the following pages the spermatogenesis of one of these forms, namely, *Cancer magister*, is described.

2. Material and Methods.

As already mentioned, the material from which these studies were made consisted of the testis of the common edible crab of the Pacific coast, *Cancer magister*. This Brachyuran is widely distributed along Puget Sound, and an abundance of material was available for study. Most of this was gathered in the vicinity of the Puget Sound Biological Station, Friday Harbor, Washington, during the summers of 1915 and 1916. In the latter part of June and the early portion of July, the testis of *Cancer magister* is in the best condition for the study of spermatogenesis. At these times every stage in the spermatogenesis process may be found within the male gonads.

The author worked almost exclusively with smears of the testicular cells. Sectioned material was also used for comparison and checking up results. The smears, however, were of greatest service and virtually all the deductions and illustrations were made from them. The manner in which the smears were prepared was as follows. Small pieces of the fresh testis were quickly mashed between two slides and immediately fixed in Bouin's fluid for about ten minutes. The slides were then washed in water until all traces of the yellowish picric acid were removed. They were next stained by the iron-alum haematoxylin method, with a counterstain of acid-fuchsin. Finally, they were run up in the usual manner through the alcohols into xylol and when fully cleared were mounted under cover-glasses with Canada balsam. Some of these preparations were unsurpassed for details regarding synapsis, chromosome numbers,

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spermatid transformations, and steps in the opening of the spermatozoa. With the exception of Figs. 1–15 and Figs. 67–69, all the drawings in the accompanying plates are from smear preparations. Figs. 1–15 are from sections, while Figs. 67–69 are from living spermatozoa as viewed in the crab's body fluids.

The material for sectioning was cut into small pieces and fixed with numerous fluids. The best fixatives, however, were found to be Flemming's strong and the Meves-Duesberg modified Flemming. The sections were cut 5μ in thickness and then stained by either the iron-alum hæmatoxylin method with a counterstain of acid-fuchsin or by the safranin, gentian-violet method.

The living spermatozoa were studied under the oil-immersion lenses in various fluids, such as the body fluids of the crab, sea water, in isotonic and hypotonic solutions of various salts (NaCl, KCl, $CaCl_2$, $NaNO_3$ and KNO_3), and in distilled water. By fixing the spermatozoa on the slide with either osmic acid fumes or Bouin's fluid, these structures could then be stained in iron-hæmatoxylin and acid-fuchsin and all the stages in their explosion could subsequently be studied.

3. Description of Testis.

The testis of *Cancer magister* has already been described elsewhere (Fasten, '15). It is a bilobed organ lying in the cephalothoracic region, below the cardiac chamber and above the digestive glands. Each lobe is profusely tubular and runs laterally along the stomach. During June and July these testicular lobes are prominently developed and fill up a large part of the upper cavity of the cephalothorax. Below the anterior portion of the heart the two lobes of the testis unite, and from this junction point two stoutly convoluted tubes, the vasa deferentia, originate and run posteriorly to the base of the fifth pair of walking legs where they open to the outside.

During the latter part of June and the early part of July the tubules in the outer and middle regions of each testicular lobe are usually undergoing rapid proliferation. Some of them are filled with spermatogonia, while others contain growth stages and still others show primary and secondary spermatocyte

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divisions. Also, many of the tubules show spermatids transforming into spermatozoa, although the mature spermatozoa are mainly confined to the tubules located near the inner or median portion of the testicular lobe as well as in the spermatophores of the vasa deferentia.

When sections of the tubules are examined, many of them are observed to be constructed on the same plan as those of *Menippe mercenaria* which were described and pictured by Binford ('13). Two or three well-defined zones may oftentimes be distinguished in one tubule. For instance, at one pole in the sectioned tubule there might be a thin layer of spermatogonia and the rest of the tubule might contain either transforming spermatids, or mature spermatozoa, or primary spermatocyte divisions, or those of the secondary spermatocyte stage, or even growth stages. When there are three zones in the tubule these are often made up of (1) a thin region of spermatogonia at one end, (2) a middle portion filled with transforming spermatids, and (3) a region of mature spermatozoa filling in the opposite pole.

The mature spermatozoa, when they enter the vasa deferentia, are surrounded by pouches known as spermatophores (Fig. 67). These spermatophores are formed by the secretions of the inner layer of epithelium which lines the vas deferent tube. For a more detailed description of these structures see Fasten, '17. During copulation the spermatophores are discharged from the vasa deferentia of the male and are deposited within the seminal receptacle of the female, where they remain dormant until the ova are mature for fertilization.

4. Spermatogenesis.

A. *Spermatogonial Stages.*

The spermatogonial cells generally line one end of the tubule. They are fairly large cells, with distinct cytoplasmic outlines and prominent nuclei. In *Cancer magister*, primary and secondary spermatogonial stages can be distinguished (Figs. 1–8). Of the two, the primary spermatogonium (Fig. 1) is somewhat larger in size, and from it by division (Figs. 2–7), is derived the secondary spermatogonium (Fig. 8).

The resting primary spermatogonium (Fig. 1) contains numer-

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ous large chromatin clumps within the nucleus. In some instances a number of linin strands could also be distinguished. The cytoplasm is more or less uniform throughout, but in a few cases large heavily staining masses, surrounded by clear outlines (Fig. 2), could be observed within it. A centrosome is almost always discernible.

When the cell begins to divide the large chromatin clumps of the nucleus undergo fragmentation (Fig. 2). This process continues until the chromatin becomes organized into a great number of heavily staining elliptical or oval structures distributed throughout the nucleus. The spermatogonium at this stage presents the picture shown in Fig. 3. Counts of these chromatin clumps were attempted, but they were found to vary considerably, ranging everywhere from forty to sixty-five. Binford ('13) has made similar observations in *Menippe mercenaria* and in the American crayfish *Cambarus virilis*, I ('14) have found the same sequence of events. Binford regards these chromatin clumps as the chromosomes which enter the equatorial plate of the spermatogonial metaphase. While these through a further fragmentation, undoubtedly form the ultimate number of chromosomes found during the metaphase stage of the spermatogonial division, yet I am inclined to the view that many of these chromatin clumps represent more than single chromosomes. This conclusion was arrived at after examination of numerous polar views of the chromosomes in metaphase plates of primary spermatogonia, in which over one hundred chromosomes could be counted. Furthermore, these chromosomes were smaller than the chromatin clumps shown in Fig. 3.

The nuclear wall surrounding the chromatin clumps soon breaks down and the cell enters the metaphase stage (Fig. 4). The chromosomes in the equatorial plate are dumb-bell shaped and the spindle fibers are rather delicate in appearance. The centrosomes can be easily distinguished at opposite poles. In polar views of sections of the equatorial plate, the chromosomes are observed to be distributed throughout the whole plane of the equator. They are small and spherical in appearance, and are so numerous that their exact number could not be determined. As already mentioned above, in many instances over one hundred

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of them could be counted. But on the assumption that there are twice the number of spermatogonial chromosomes as are found in the reduction division, there must be one hundred and twenty chromosomes in the spermatogonial stages of *Cancer magister*.

The anaphase (Fig. 5), and telephase (Figs. 6 and 7) stages follow each other quickly, thus dividing the cell into secondary spermatogonia (Fig. 8). These are somewhat smaller than the primary spermatogonia (compare Figs. 1 and 8), and in the resting condition (Fig. 8), the chromatin of their nuclei stains much more heavily. Otherwise, the secondary spermatogonia resemble the primary ones markedly and their division proceeds in exactly the same fashion. The ultimate divisions of the secondary spermatogonia produce the resting primary spermatocytes (Fig. 16).

In many spermatogonial strips of the tubules, some of the cells were found to be undergoing degeneration. In such cases, the cells lose their distinctness of outline and their nuclei come to lie in a syncitial mass of protoplasm. In many instances, the nuclei resemble those of the spermatogonia (compare Fig. 9 with Fig. 1); in others (Figs. 10–12), the nuclei become transformed into very large irregular structures with pseudopodia-like projections. These cells are the so-called "nutritive cells" and they may be best studied in tubules where mature spermatozoa are found. In *Cambarus virilis*, I ('14) have noticed similar cells.

The nutritive cells (Figs. 9–15) are very interesting structures. Their nuclei contain heavily staining chromatin masses, while the cytoplasm possesses numerous fat globules which stain intensely black with osmic acid. Many of the earlier investigators on the Decapoda such as Grobben ('78), Gilson ('86), and Herrmann ('90) have claimed that the spermatogonia are derived from the nutritive cells. On the other hand, St. George ('92), and Keppen ('06) claim the opposite, that the nutritive cells are derived from a transformation of the spermatogonia. This last-mentioned condition seems to be the case in *Cancer magister*.

When sections of the nutritive cells are examined, numerous stages like those seen in Figs. 13–15 may be observed, which

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strongly suggest amitosis. The fact is that the earlier workers on the Decapoda all claim such division in the nutritive cells. However, a careful study of the nuclei shows many of them to be amboeoid in appearance (Figs. 10–12), and this being the case, it is entirely possible to derive the stages represented in Figs. 13–15 from sections through such cells as are shown in Figs. 10–12. It is, therefore, very difficult as well as dangerous to come to any certain conclusions concerning amitosis from sectioned material.

B. *Primary Spermatocyte Stage.*

This stage follows the spermatogonial divisions. After a period of growth and synapsis, reduction occurs. During the growth period two definite spherical bodies, surrounded by clear spaces, make their appearance in the cytoplasm. These are the so-called chromatoid bodies, and they appear to be similar to the same structures which I ('14) have previously described in *Cambarus virilis*.

(*a*) *Growth Period.*—This period includes the preparatory stages, synapsis and tetrad formation. During the early prophases the chromatin in the nucleus of the resting primary spermatocyte (Fig. 16), consists first of a few large, heavily staining clumps, but these soon undergo a fragmentation process, whereby smaller chromatin masses (Fig. 17) are produced. In a few cells, linin threads which were rather indistinct and granular in appearance were observed. Sometimes large chromatoidal masses like those shown in Fig. 17 were seen within the cytoplasm.

The chromatin of the nucleus breaks up into still smaller structures and these then begin to weave out into thin leptotene threads (Fig. 18). At the same time the cell increases somewhat in size (compare Figs. 16 and 17 with Fig. 18). This stage, represented by Fig. 18, really marks the beginning of the growth period and from now on the increase in the size of the cells is more evident. When Fig. 18 is examined it can be seen that no continuous spireme is formed. The leptotene threads remain separate and may be distinguished from each other. Owing to the great number of these threads it was impossible to count them.

The primary spermatocytes now undergo synizesis. The leptotene threads migrate to one pole of the nucleus (Fig. 19),

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the so-called "synaptic pole" and arrange themselves in parallel pairs. This paired arrangement is very distinct and has been observed in a great many cells. While this is going on, two large round bodies, of more or less equal size, and surrounded by clear spaces (Fig. 19, *k*) make their appearance in the cytoplasm. These bodies were first observed in a smear preparation which was heavily stained. They stained exactly like the chromatin. In preparations which were strongly destained no trace of them was found. However, when these latter preparations were restained, the bodies loomed up with exceptional clearness. It is thus seen that although these structures stain like chromatin, yet they differ from chromatin in their affinity for nuclear dyes, and, in all probability, they are chemically different from chromatin. Wilson ('13), first described a similar body in *Pentatoma*, and called it a "chromatoid body." In *Cambarus virilis*, I ('14) have found a pair of chromatoid bodies appearing at about the same stage in the developing spermatocyte as in *Cancer magister*. After the leptotene stage, the chromatoid bodies of *Cancer magister* persist within the cells, and their subsequent history will be outlined when the respective stages of the maturation are dealt with.

Synapsis soon sets in. The parallel threads become more closely paired at the synaptic pole and the cell enters the pachytene stage, in which the pairs of leptotene threads fuse into thick gemini (Fig. 20). Here the union appears complete as no traces could be found of the longitudinal furrows which separated the original pairs of parallel threads.

After remaining fused for some time, the components of each geminus begin to unravel. Along every geminus a longitudinal split makes its appearance (Fig. 21), and simultaneously with this there is an opening of the paired threads at one end, while remaining attached at the other end, thereby producing figures which appear like 8, V, or less commonly like U (Figs. 21 and 22).

Another longitudinal split soon occurs along the arms of each geminus. In Fig. 22 the beginnings of this second longitudinal furrow are clearly visible in the two arms of the 8 which is located in about the center of the nucleus. By means of these two longitudinal cleavage planes, four chromatin threads are formed,

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distinctly opened up at one end and temporarily fused at the opposite end. The two pairs of these threads continue to diverge in opposite directions until they ultimately form X's (Fig. 23).

The pairs of threads on opposite sides of the central fusion point of each X now migrate closer to each other until they come to lie almost parallel. The central fusion point next disappears, and four thin threads arranged in two parallel rows are produced. A hasty glance at this arrangement makes it appear as if the geminus from which the four threads were derived, was traversed by a longitudnal and a transverse cleavage plane (see Fig. 23). However, prolonged and careful study reveals the true nature of the case, that the threads under discussion are the results of two longitudnal splits of each geminus.

The tetrads are soon formed. Each thin thread shortens and thickens into a spherical chromatin mass (Figs. 23 and 24), so that very soon every geminus is changed into four spherical chromosomes, representing a tetrad (Figs. 23–25). Each tetrad thus contains four univalent chromosomes. In the next step pairs of these univalent chromosomes fuse, resulting in two large bivalents attached to each other by linin strands. This condition is particularly well shown in Figs. 24 and 25. The condensation of the bivalents continues until they are changed into dumb-bell shaped structures (Fig. 25). The growth process is now completed and the cell is ready to undergo reduction.

It is quite evident from the above discussion that in *Cancer magister* we apparently have to deal with a case of parasynapsis, or side by side conjugation of the chromosomes. This conclusion was reached after prolonged observations upon numerous excellent smear preparations, which contained an abundance of synapsis material. The essential steps closely resemble those of *Cambarus virilis* (Fasten, '14).

(*b*) *Reduction Division.*—In the last stages of the growth period, the nuclear wall begins to break down (Fig. 24). The two centrosomes, which have been formed by a division of the original centrosome, migrate to opposite poles, and thin spindle fibers make their appearance between them and the chromosomes. The chromosomes are soon pulled to the equator of the cell to undergo reduction. In Fig. 25, the dumb-bell shaped bivalents

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are seen arranging themselves. Here the chromatoid bodies can also be clearly discerned at opposite poles. Each of these bodies is surrounded by its characteristic clear space and may be easily recognized from the ordinary chromosomes.

In the metaphase period (Fig. 26), all of the chromosomes are grouped in the equatorial plane and appear as large dumb-bells. When the component bivalents of each dumb-bell are examined, they are found to be large spherical bodies, about twice the size of the spermatogonial chromosomes. Furthermore, these bivalents do not show the equational furrows where the equational division of the second spermatocyte will take place. Binford ('13), in describing this stage in *Menippe mercenaria* claims that the chromosomes in the equatorial plate of one of his preparations were found to be ring-like in appearance. In other preparations which were destained to the degree of removing all the stain from the cytoplasm and the achromatic figure of the metaphase, this same investigator asserts that he could distinguish the individual four chromosomes of each tetrad. In *Cancer magister* no such results were obtained in spite of the fact that a great many smears and sections were carefully examined.

In polar views of the metaphase stage sixty chromosomes have been distinguished (Figs. 27 and 28). These are generally oval in shape; some of them being larger than others and they are distributed throughout the equatorial plane. Figures 27 and 28 are drawings of polar views as seen in smear preparations. Similar counts of polar views in sectioned material have corroborated this number for the reduction division.

The chromatoid bodies always migrate undivided to opposite poles of the cell. In many cases they occupy positions along the spindle fibers (Figs. 26, 29 and 30), while in other cases they may be seen in the cytoplasm (Figs. 31 and 32). When they occupy positions along the spindle fibers, one would be easily misled into regarding them as accessory chromosomes, especially so if no attention were paid to the various stages of the growth period. Wilson ('13) has cited numerous cases in which investigators have undoubtedly confused chromatoid-like bodies with accessory chromosomes. After citing these instances, Wilson, on p. 403, then makes the following significant remarks:

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"Such facts make it clear that the presence of sex-chromosomes can not safely be inferred alone from the presence of chromosome-like bodies lagging on the spermatocyte-spindles, or lying near one pole. The presence of compact, deeply staining nucleoli during the growth-period is by itself equally indecisive. In some cases the 'plasmosome,' especially after certain fixatives such as Bouin's fluid, may stain quite as intensely as the chromosome-nucleoli with haematoxylin, safranin and other dyes (cf. Gutherz, '12). Decisive evidence regarding these bodies can only be obtained by tracing their individual history and by accurate correlation of the chromosome-numbers in the spermatogonial and spermatocyte-divisions. It hardly need be added that great caution is necessary in dealing with difficult material in which for any reason such a test cannot be completely carried out."

The anaphase stage (Fig. 29) follows upon the metaphase. The bivalents of the dumb-bells are separated and pulled to opposite poles. The chromatoid bodies also migrate in these directions. The division process continues and gradually the primary spermatocyte enters the telophase stage (Figs. 30 and 31). In the final telophase (Fig. 32), the chromosomes which have completely migrated to opposite poles become surrounded by thin nuclear walls. The cytoplasm has constricted off into two distinct portions and during this process the spindle fibers have also been constricted so that at their center a thickened "zwischenkörper" is formed. When this stage is completed two secondary spermatocytes are produced, each possessing one of the chromatoid bodies within the cytoplasm (Fig. 32).

C. *Secondary Spermatocyte Stage.*

The secondary spermatocytes formed during the ultimate telophase stage of the reduction division, undergo immediate transformations. No rest period could be determined. This is similar to what occurs in *Astacus fluviatilis* (Prowazek, '02), in *Menippe mercenaria* (Binford, '13), and in *Cambarus virilis* (Fasten, '14). The cells assume the metaphase stage (Fig. 33), and the chromosomes line up in the equator of the spindles in the form of dumb-bells. When polar views of these chromosomes are studied in preparations which have been greatly destained,

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sixty of them may be counted (Figs. 34 and 35). These chromosomes are about half the size of those found in the reduction division (compare Figs. 34 and 35 with Figs. 27 and 28). As for the chromatoid body, it generally lies at or near one pole of the cell, leaving the other pole without any such element.

The anaphase (Fig. 37) and telophase (Figs. 38 and 39) stages follow each other in logical sequence, resulting in the division of the secondary spermatocytes to form spermatids. Two types of spermatids (Fig. 40), are thus formed in equal numbers; one type which contains a single chromatoid body in the cytoplasm (Figs. 40 and 41), whereas, the second type is without this body (Figs. 40 and 43).

D. *Transformations of the Spermatids into Spermatozoa.*

The spermatids produced are, at first, small and their nuclei contain large masses of chromatin material which stain intensely with nuclear dyes (Fig. 40). The cytoplasm is homogeneous throughout and within it a rather prominent centrosome is found. In the second type of spermatid developed, the cytoplasm, in addition to containing the centrosome, also possesses the chromatoid body (Figs. 40 and 41).

The first noticeable changes undergone by the spermatids in transforming into spermatozoa, occur in the nucleus. The chromatin mass of the nucleus is gradually reduced to such a degree that it loses its intense staining qualities, and becomes quite homogeneous in consistency (Figs. 40–47). At first the large chromatin clumps break up into granular masses (Figs. 40, 43 and 44). Then these fragment still more completely until in the final stages there remain, respectively, three round chromatin bodies (Fig. 45); then two (Figs. 42 and 46), and ultimately one (Fig. 47). This remaining chromatin structure is spherical, stains intensely black with Heidenhain's haematoxylin and occupies the center of the nucleus. It may be said to be a nucleolus-like body which resembles a karyosome.

Both classes of spermatids produced undergo similar changes of the nucleus. In the second type of spermatid, however, an interesting change goes on in the cytoplasm, simultaneously with the transformations of the nucleus. Here the chromatoid body

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wanders from its position within the cytoplasm to the periphery of the cell and is soon expelled to the outside (Figs. 40–42), thus playing no further rôle in the transformations. From now on all the spermatids are exactly alike and they undergo the same modifications.

At about this time a densely staining mass makes its appearance in the cytoplasm (Figs. 46 and 47, *m*). This mass has been called a mitochondrial mass by Koltzoff ('06) and Binford ('13). It stains like the chromatin of the nucleus, and first makes its appearance at about the stage in the spermatid transformations where the chromatin of the nucleus becomes reduced to two clumps (Fig. 46). As to whether this mass consists of mitochondria or not is a debated question. The fact of the matter is that cytologists themselves are not clear as to which bodies within the cell are mitochondria and which are not (see Cowdry, '16). In the cells under consideration, no traces of mitochondria have been observed in the earlier stages of the maturation. This darkly-staining mass makes its appearance only after most of the chromatin within the spermatid nucleus has been much reduced. This would lead one to suspect that the mass might consist of chromatin which has diffused out of the nucleus and has accumulated within the cytoplasm (see Figs. 43–47). At any rate, this seems a likely probability.

The spermatids now reveal the following distinct structures, (1) a rather homogeneous nucleus (Fig. 47, *n*), with a karyosome-like body in the center, (2) a cytoplasm in which are found (3) a centrosome (Fig. 47, *c*), and (4) a mitochondria-like mass (Fig. 47, *m*), which stains like chromatin. These elements must be clearly kept in mind in order to follow up the later changes of the spermatids. In linking up these changes it is also necessary to exercise great care. Sectioned material, while helpful, is by itself wholly inadequate for this purpose. Smear preparations of the entire cells, on the other hand, give a true picture of what happens, and these were used almost entirely in the study of transformations.

The nucleus wanders to one pole of the spermatid (Figs. 48–50, *n*), while at the opposite pole a clear vacuole (Fig. 50, *v*),

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makes its appearance. Sometimes two clear openings (Figs. 48–49, *v*) may be seen, but these later flow together into a single one (Fig. 50, *v*). At the same time the mitochondrial mass wanders in between the nucleus and the vacuole (Figs. 48–52, *m*), and ultimately fills this entire space (Fig. 53). The centrosome increases somewhat in size and takes a position in the center of the mitochondrial mass (Figs. 50–53, *c*).

The mitochondrial mass now transforms into a ring resembling a doughnut, and the centrosome comes to occupy the center of its inner open space (Figs. 53 and 54, *c*). The upper portion of the nucleus also becomes located in this space (Figs. 54–58, *n*). At the same time, the karyosome-like body, situated within the center of the nucleus (Figs. 47–53) migrates upward to the middle of the upper portion of the nucleus (Fig. 54) until it comes to lie directly below the centrosome.

Binford ('13) in describing the transformation of the spermatids of *Menippe mercenaria* claims, on p. 156, that "after the mitochondrial ring is completed the nucleus becomes widely separated from it and the capsule (Figs. 50 to 52). This, however, is not always the case. In two preparations from which Figs. 33 to 35 and 37 to 43 were drawn, the nucleus remained fitted closely on the capsule as shown in Fig. 43. As the two different conditions were obtained with the same fixing fluid it is hardly probable that the difference was caused by the fixing."—In sections of testicular material of *Cancer magister*, a few of the transforming spermatids showed the conditions which Binford describes, but in smear preparations not a single such instance was discernible. I therefore suspect that Binford had to deal with defects which are often produced in cytological material which is prepared by the fixation and the sectioning methods.

Simultaneously with the last-mentioned changes, a second vacuole (Fig. 54, *v'*), makes its appearance in the anterior extremity of the original first (Fig. 54, *v*), or primary vacuole. At first this second vacuole looks like a small bubble of liquid which stains rather darkly with Heidenhain's haematoxylin. It soon increases in size, becomes more distinct (Figs. 54–57, *v'*), and stains somewhat lighter. It is quite evident that Binford's ('13) so-called "inner tubule" formed during the spermatid

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transformations of *Menippe mercenaria*, is the same structure which I have called the second vacuole in *Cancer magister*. My preparations, however, show no such stages which Binford describes and pictures (Figs. 52–60 of Binford, '13), for the development of this structure.

During this time the centrosome and the karyosome-like body of the nucleus unite (Figs. 54–56), and elongate into a rod-like structure (Figs. 57 and 58), the so-called central body (Figs. 56 and 57, *b*). At first the central body looks like a dumb-bell (Figs. 56 and 57, *b*), but as it lengthens out, it loses this appearance, becomes more rod-like (Figs. 58–60), and at the same time penetrates the inner or proximal portion of the second vacuole. While all these changes are going on the primary and secondary vacuoles are gradually transforming into primary and secondary vesicles (see Figs. 54–60, *v* and *v'*), and from now on they will be designated by the latter names.

At about the stage represented by Fig. 59, an opening makes its appearance in the middle of the outer, or distal end of the second vesicle. Simultaneously with this, the central body elongates still more (Fig. 59) and its outer extremity seems to hollow out into a thin tube (Figs. 60–62) which soon connects up with the distal opening in the secondary vesicle. As the outer end of the central body hollows out, a ring of densely staining material makes its appearance around the outer opening of the second vesicle (Figs. 61–63, *d*). In Heidenhain's haematoxylin this ring stains intensely black like the centrosome or the chromatin. This ring may be spoken of as a chromatin-ring and it becomes more distinct as the distal end of the central body hollows out (Figs. 61–64, *d*). Going hand in hand with these modifications are those which take place in the mitochondria-like ring and the nucleus. These two elements fuse into a single structure, a sort of nuclear-mitochondrial cup (Fig. 62, *h*), which stains quite homogeneously, although some part of the mitochondrial mass seems to persist around the wall of the first vesicle and stains more intensely than the rest of the cup. (see Fig. 62, *h*).

As the transformations continue, the second vesicle fits more compactly into the first vesicle (Figs. 63 and 64). The outer portion of the central body has completely changed into a thin-

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walled hollow tube, which stains lightly. The inner portion of the central body is solid, stains intensely with nuclear dyes and is embedded in the center of the nuclear-mitochondrial cup, immediately below the wall of the first vesicle. Figs. 63–65 show these structures nicely. Now the radial arms or rays of the spermatozoön (Figs. 63 and 64, *r*) make their appearance. They originate as outgrowths from the nuclear-mitochondrial cup, and in the finished state they are stout structures with pointed extremities (Figs. 63 and 64, *r*).

Soon the spermatids are completely transformed into mature spermatozoa, and in this state (Figs. 65 and 66) they look like ovoid, or spheroid structures, in which the radial rays are tightly coiled around the nuclear-mitochondrial cup. Figs. 65 and 66 are drawings of mature spermatozoa as viewed, respectively, from the side and bottom. An examination of these figures shows the central body (*b*) located in the middle, and surrounding it in order of sequence are the second vesicle (*v'*), the first vesicle (*v*) and the nuclear-mitochondrial cup (*h*). In Fig. 65, the structure of the central body (*b*) consisting of a hollow distal end and a solid proximal end, can be distinctly observed. In this figure may be also seen the densely staining chromatin-ring (*d*), located at the upper end of the secondary vesicle.

When the mature spermatozoa are studied in smear preparations which have been fixed with Bouin's fluid and stained by the iron-haematoxylin and acid-fuschin methods, then the second vesicle stains a dark amber color, while the primary vesicle takes almost no stain and remains clear. In sections fixed with Flemming's fluid and stained similarly, the second vesicle remains more or less transparent, while the first vesicle stains a dark brown.

The mature spermatozoa are at first free, but when they make their way into the vasa deferentia they are surrounded by the membranous pouches or spermatophores already mentioned under the discussion of the testis. Fig. 67 shows one of these spermatophores when it is first removed from the vas deferens of the living crab. Notice the numerous spermatozoa which are compactly stored within its interior.

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5. Opening of the Mature Spermatozoa.

The mature spermatozoa within the spermatophores are dormant structures, but when they are removed from the spermatophores and placed in fluids whose concentration is less than sea water, they undergo interesting changes. In many ways these are similar to the changes which Binford ('13) describes for the spermatozoa of *Menippe mercenaria*.

The method employed in studying the opening of the mature spermatozoa of *Cancer magister* was similar to that employed by Binford ('13). Numerous spermatophores suspended in either crab's body fluids, or sea water, were placed on a slide and covered with a cover-glass. By applying pressure to the cover-glass many of the spermatophores were ruptured, thus liberating the spermatozoa. These could then be examined under the high powers of the microscope. By allowing numerous salt solutions, already mentioned under the section on 'Materials and Methods,' to diffuse under the cover-glass, all of the changes in the opening up process or the so-called explosion of the spermatozoa could be followed out quite accurately. Many of these spermatozoa in various stages of their explosion were fixed and stained on the slides and were then used for later study and comparison.

In the crab's fluids, in sea water, or in solutions of NaCl, KCl, $CaCl_2$, $NaNO_3$ and KNO_3 which are isotonic with sea water, almost no change occurs. Usually the rays of the nuclear-mitochondrial cup unravel, revealing spermatozoa which contain either three (Fig. 68) or four (Fig. 69) rays. In hypotonic solutions of the last-mentioned salts, the spermatozoa undergo an explosion, and change considerably in appearance. Osmotic pressure, undoubtedly, accounts for this explosion as was suggested by Koltzoff ('06).

The first step in the explosion of the spermatozoa is the extrusion of the second vesicle. This vesicle normally surrounds the central body and is embedded in the first vesicle (Figs. 70 and 71). When the second vesicle begins to extrude, it swells somewhat (Figs. 72–76, *v'*) in size and at the same time it stretches the upper portion of the first vesicle and makes it appear like a thickened ring (Figs. 72–76, *s*). Simultaneously with this, the

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hollow distal end of the central body which is also extruded, exerts a pull on its solid proximal portion, transforming it into a spine-like structure (Figs. 72–76, *b*), which stains intensely black with Heidenhain's hæmatoxylin. While these changes are going on, the nuclear-mitochondrial cup loses its rays and rounds out into a spherical body.

When the second vesicle has been completely extruded (Fig. 75, *v'*), then the first vesicle (Fig. 75, *v*) commences to evert and continues this process until it is completely turned inside out. These steps may be observed in Figs. 75–79, *v*. During the eversion, the darkly staining proximal end of the central body forces upward on its tubular distal portion until the latter is finally extruded completely to the outside (see Figs. 75–77, *b*).

The completely exploded spermatozoa present the appearances represented in Figs. 78 and 79. The upper portion consists of the second vesicle (*v'*), in the interior of which is contained the everted first vesicle (*v*), with its upwardly projecting spine-like body (*b*). The lower portion consists of the nucleo-mitochondrial cup (*h*), which has transformed into a more or less spherical structure. In many cases, stages like those shown in Fig. 80 were seen. These evidently are exploded spermatozoa in which the second vesicle has completely ruptured and disintegrated. Binford ('13) has observed similar conditions in *Menippe mercenaria*.

6. Discussion.

A. *Synapsis*.

During the last few years the parasynaptic view of chromosomal conjugation has been established in numerous species of animals. In 1900, Von Winniwarter first advocated parasynapsis amongst the mammals, but the view did not become firmly established until the Schreiners ('04, '05, '06, '07 and '08) published their important researches on the germ cells of many animals including mammals, birds, reptiles, amphibians, fish, molluscs and annelids. Of late the parsaynapsis view of Von Winniwarter and the Schreiners has been extended to a great many additional forms, and excellent reviews of the vast literature on this subject may be found in the recent publications of Montgomery ('11), Wilson ('12), Fasten ('14) and Wenrich ('16).

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Among the Crustacea, parasynapsis has been established in some of the Copepoda (Lerat, '05; Matschek, '09; McClendon, '10; and Kornhouser, '15), and also in one of the Decapoda (Fasten, '14). In the decapod crustacean *Cambarus virilis*, I ('14) showed that during the growth period, the chromosomes conjugate in parasynaptic fashion, and as already pointed out elsewhere in this paper, this is the type of conjugation which occurs in *Cancer magister*. In both *Cancer magister* and *Cambaras virilis*, the great difficulty encountered in the study of synapsis was the immense number of chromosomes. However, after prolonged and careful study of the various stages in the growth period of these animals, one finds it rather difficult to interpret the conjugation of the chromosomes in any other way than by parasynapsis.

B. *The Chromatoid Bodies.*

Ever since Wilson ('13) called attention to a chromatoid body in the spermatogenesis of *Pentatoma*, similar structures have been described during the spermatogenesis stages of other forms. In *Cambarus virilis* (Fasten, '14), two such bodies were found which could be traced into the spermatid stages and then all traces of them were lost. In *Cancer magister*, a pair of chromatoid bodies make their appearance during the synizesis stage of the growth period, and during the reduction division these pass to opposite poles, so that the secondary spermatocytes each possess a chromatoid body. During the equation division, this body passes undivided to one pole, resulting in two types of spermatids, one type possessing a chromatoid body, while the other type is minus such a structure. It is also of interest that the chromatoid body is eventually expelled from the first type of spermatid thus playing no further part in spermatogenesis.

Concerning the nature and function of the chromatoid body, very little can be said. In only two forms, namely *Pentatoma*, (Wilson, '13) and in the decapod under consideration *Cancer magister*, has the full history of this structure been traced, and in both cases, it is expelled from the spermatids, thus appearing to play no definite rôle in the mature spermatozoa. Wilson ('13), in discussing the chromatoid body, makes the following

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trite remarks on page 402: "The nature of the chromatoid body thus remains problematical, but the facts are worthy of serious attention for another reason. Were the chromosomes very small, numerous, closely crowded, or otherwise unfavorable for exact study, and could not the entire history of the chromatoid body be so clearly traced, even an experienced observer might fall into the most confusing error concerning the relations of the chromosomes."

7. Summary.

1.. During the latter part of June and the early part of July the testicular lobes of *Cancer magister* are in the best shape for the study of spermatogenesis.

2. Two spermatogonial divisions can be recognized, and these ultimately form the resting primary spermatocytes.

3. Sometimes, larger and more intensely staining cells are found interspersed among the spermatogonia. These are the nutritive cells, and it seems very probable that they have originated from a transformation of some of the spermatogonia. The nuclei of the nutritive cells are irregular in shape and many of them possess amœboid processes. In sections of some of the nutritive cells two or more nuclei are oftentimes found, and this might easily mislead one into concluding that amitosis occurs amongst them.

4. The resting primary spermatocyte undergoes a growth period, during which thin leptotene threads are produced through the fragmentation of the chromatin. No continuous spireme is formed as the leptotene threads appear distinct and separate.

5. During the growth period pairs of leptotene threads migrate to the synaptic pole of the cell, become arranged in parallel fashion and soon fuse parasynaptically.

6. During the synizesis stage of the growth period a pair of densely staining chromatoid bodies make their appearance in the cytoplasm. These are surrounded by clear areas and may have originated from some of the chromatoid masses found within the cytoplasm of some of the earlier stages in the spermatogenesis.

7. The first spermatocyte division is reductional. In the metaphase stage the chromosomes line up as dumb-bells, which are composed of pairs of bivalents. The chromatoid bodies pass undivided to opposite poles of the cell.

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8. A polar view of the metaphase stage of the reduction division reveals sixty chromosomes distributed throughout the entire plane of the equator.

9. The division of the primary spermatocytes results in secondary spermatocytes, each of which contains a chromatoid body.

10. The second spermatocyte division is equational and immediately follows the reduction division. A polar view of the metaphase stage of the equational division reveals sixty chromosomes which are about half the size of those found during the reduction division.

11. The chromatoid body passes undivided to one pole during the division of the secondary spermatocyte, resulting in the formation of two classes of spermatids, one of which contains the chromatoid body, while the other is without such a structure.

12. The chromatoid body is soon expelled from the spermatids which contain it, thus making all the spermatids alike in structure and appearance.

13. The nucleus of the spermatid loses its large quantity of intensely staining chromatin, while at the same time a mitochondria-like mass makes its appearance in the cytoplasm. Also one or two vacuoles are formed in the cytoplasm.

14. As the transformations go on the nucleus becomes elliptical and wanders to one pole of the cell. The vacuoles fuse into a single large vacuole which then takes a position at the opposite pole of the cell. The mitochondria-like mass wanders in between these two structures, becomes ring-like, and within its center and above the karyosome-like body of the nucleus, the centrosome becomes stationed. Soon a second vacuole makes its appearance at the distal end of the first one.

15. The two vacuoles gradually transform into the first and second vesicles. The centrosome and karyosome-like body of the nucleus become fused into the central body, which runs through the middle of the second vesicle, while the nucleus and mitochondria-like ring unite into a nucleus-mitochondrial cup from which the rays of the spermatozoön are produced.

16. The mature spermatozoa are oval bodies tightly packed within membranous spermatophores.

17. When the mature spermatozoa are surrounded with salt

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solutions possessing a lower osmotic pressure than either the crab's body fluids or sea water, they undergo an interesting explosion in which the vesicles and the central body are completely everted, while at the same time the nuclear-mitochondrial cup rounds out into a spherical structure.

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9. DESCRIPTION OF PLATES.

All the illustrations in the accompanying plates were made with the aid of the camera lucida. Figs. 1–66 and 70–80 were drawn under Zeiss apochromatic objective 2 mm. Hom. Im., Comps. ocular 12,[1] at a magnification of 2,720 times. Figs. 67–69 were drawn under achromatic lenses at an approximate magnification of 1,400 times.

Abbreviations.

b = central body.
c = centrosome.
d = chromatin ring.
h = nuclear-mitochondrial cup.
k = chromatoid bodies.
m = mitochondria-like mass.
n = nucleus.
r = rays or arms of spermatozoa.
v = first or primary vacuole (first or primary vesicle).
v' = second or secondary vacuole (second or secondary vesicle).

Explanation of Plate 1.

Fig. 1. Resting primary spermatogonial stage.

Fig. 2. Spermatogonial prophase, showing fragmentation of chromatin. Within the cytoplasm two large, heavily staining masses, probably chromatoid masses can be distinguished.

Fig. 3. Late spermatogonial prophase. Note the compact chromatin clumps within the nucleus.

Fig. 4. Metaphase, primary spermatogonium.

Fig. 5. Anaphase, primary spermatogonium.

Figs. 6 and 7. Telophase, primary spermatogonium.

Fig. 8. Resting secondary spermatogonial stage.

Figs. 9 to 12. Nutritive cells. Observe the irregular nuclei and the fatty globules within the cytoplasm.

Fig. 13. Nutritive cell, in which the nucleus shows a constriction in the middle which appears to be suggestive of amitosis.

[1] I wish to express my indebtedness and gratitude to the Department of Zoölogy of the University of Wisconsin for the loan of a set of Zeiss apochromatic lenses (2 mm. oil immersion objective and compensating oculars 4, 8 and 12), which were of great service in the problem under consideration.

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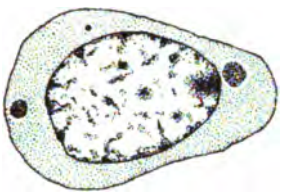

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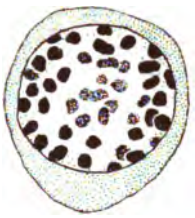

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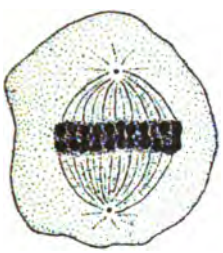

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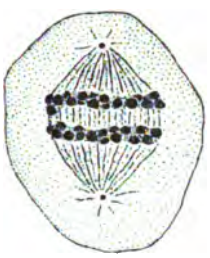

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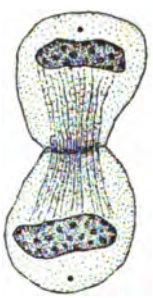

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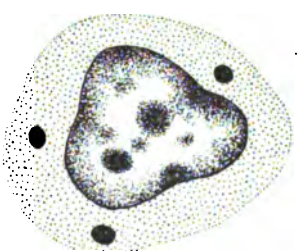

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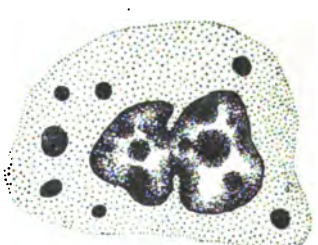

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EXPLANATION OF PLATE 2.

FIGS. 14 and 15. Nutritive cells, with two nuclei, suggestive of amitosis.

FIGS. 16 and 17. Early prophases, primary spermatocyte stage. In Fig. 17 two darkly staining chromatoid masses can be seen within the cytoplasm.

FIG. 18. Leptotene stage.

FIG. 19. Synizesis and synapsis stage. In this stage the leptotene threads have a paired parallel arrangement at the synaptic pole of the cell. The chromatoid bodies (*k*), and the centrosome (*c*), are also visible in the cytoplasm.

FIG. 20. Pachytene stage. The paired leptotene threads have united into thick gemini.

FIGS. 21 and 22. Diplotene stage.

FIG. 23. Postdiplotene stage.

FIG. 24. Tetrad formation. In this stage the nuclear wall begins to break down.

FIG. 25. Tetrads transformed into dumb-bells. The cell is entering the metaphase and the chromatoid bodies, surrounded by clear spaces are seen at opposite poles.

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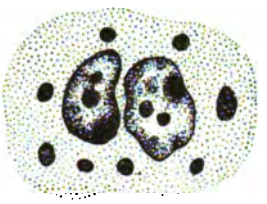

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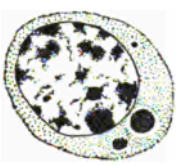

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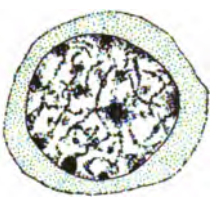

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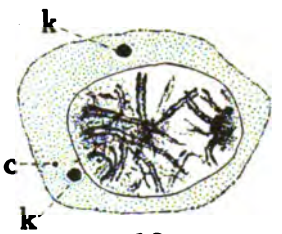

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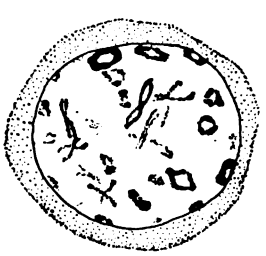

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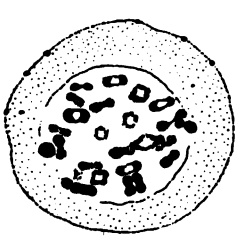

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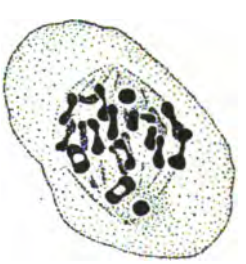

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Explanation of Plate 3.

Fig. 26. Metaphase, primary spermatocyte showing chromatoid bodies along the spindle fibers at opposite poles.

Figs. 27 and 28. Polar views, primary spermatocytes, showing 60 chromosomes.

Figs. 29 to 32. Anaphase and telophase stages of primary spermatocytes showing the chromatoid bodies at opposite poles. In Fig. 32 the chromatoid bodies are observed to remain in the cytoplasm.

Fig. 33. Metaphase, secondary spermatocyte, showing a single chromatoid body at one pole.

Figs. 34 and 35. Polar views, secondary spermatocytes, showing 60 chromosomes.

Figs. 36 and 37. Anaphase stages, secondary spermatocytes, showing different positions which the chromatoid body may occupy in the dividing cell.

Figs. 38 and 39. Telophase stages, secondary spermatocytes. The single chromatoid body is at one pole.

Fig. 40. The two types of spermatids formed, one without the chromatoid body, and the other containing it. The centrosomes are the minute dark granules found in the cytoplasm of both these types of spermatids.

Fig. 41. Early stages in the transforming spermatid which contains the chromatoid body.

Fig. 42. Stage in which the chromatoid body is expelled from the spermatid that contained it.

Figs. 43 to 47. Early transformation stages of spermatids which are minus the chromatid body. Note the reduction of the chromatin and the appearance of the mitochrondria-like mass (*m*) in the cytoplasm. In Fig. 47 the single karyosome-like body occupying the center of the nucleus may be seen.

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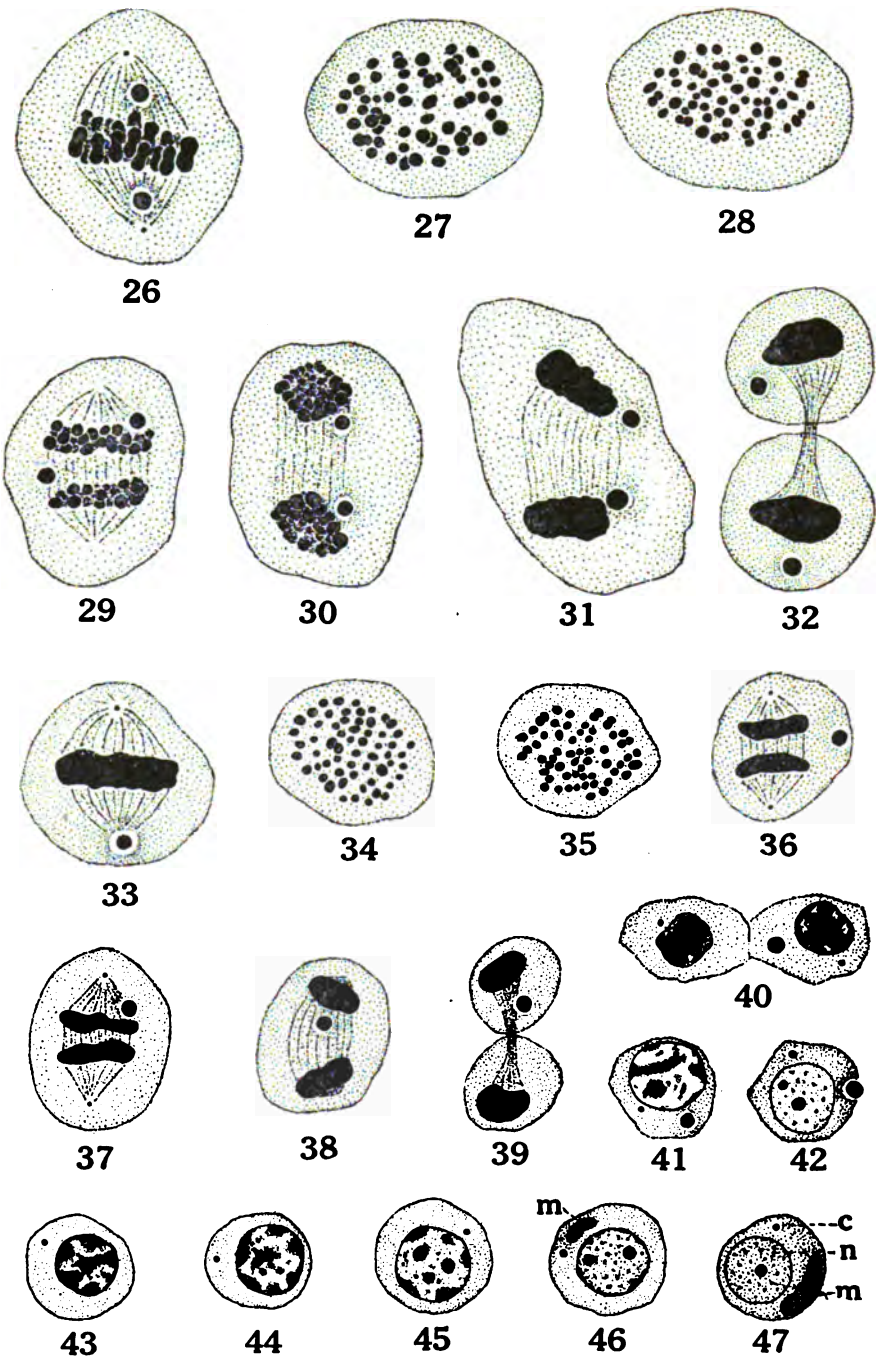

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Explanation of Plate 4.

Figs. 48 to 53. Successive stages in the transformation of the spermatid, resulting in the primary vacuole (*v*) being formed at one pole, while the nucleus (*n*) occupies the opposite pole, and in between them the mitochrondria-like mass (*m*) and the centrosome (*c*) take their position.

Fig. 54. The secondary vacuole (*v'*) makes its appearance at the distal end of the first vacuole (*v*).

Figs. 55 to 58. Stages in the transformation of the spermatid in which the primary and secondary vacuoles transform into distinct vesicles, and the central body (*b*) assumes a dumb-bell appearance.

Figs. 59 to 62. Spermatid transformations showing the hollowing out of the distal end of the central body; the formation of the chromatin-ring (*d*), and the fusion of nucleus and mitochondria-like mass into a nuclear-mitochondrial cup (*h*).

Figs. 63 and 64. Formation of the rays (*r*) of the spermatozoön.

Figs. 65 and 66. Side and bottom view of mature spermatozoa, showing details of structure.

Fig. 67. Spermatophore, filled with mature spermatozoa.

Figs. 68 and 69. Three- and four-rayed types of spermatozoa, as seen suspended in the body fluid of *Cancer magister*.

Figs. 70 to 78. Successive stages in the explosion of the mature spermatozoön when surrounded with salt solutions of lower osmotic density than sea water.

Fig. 79. Spermatozoön which has exploded in distilled water.

Fig. 80. Exploded spermatozöon in which the secondary vesicle has completely disintegrated.

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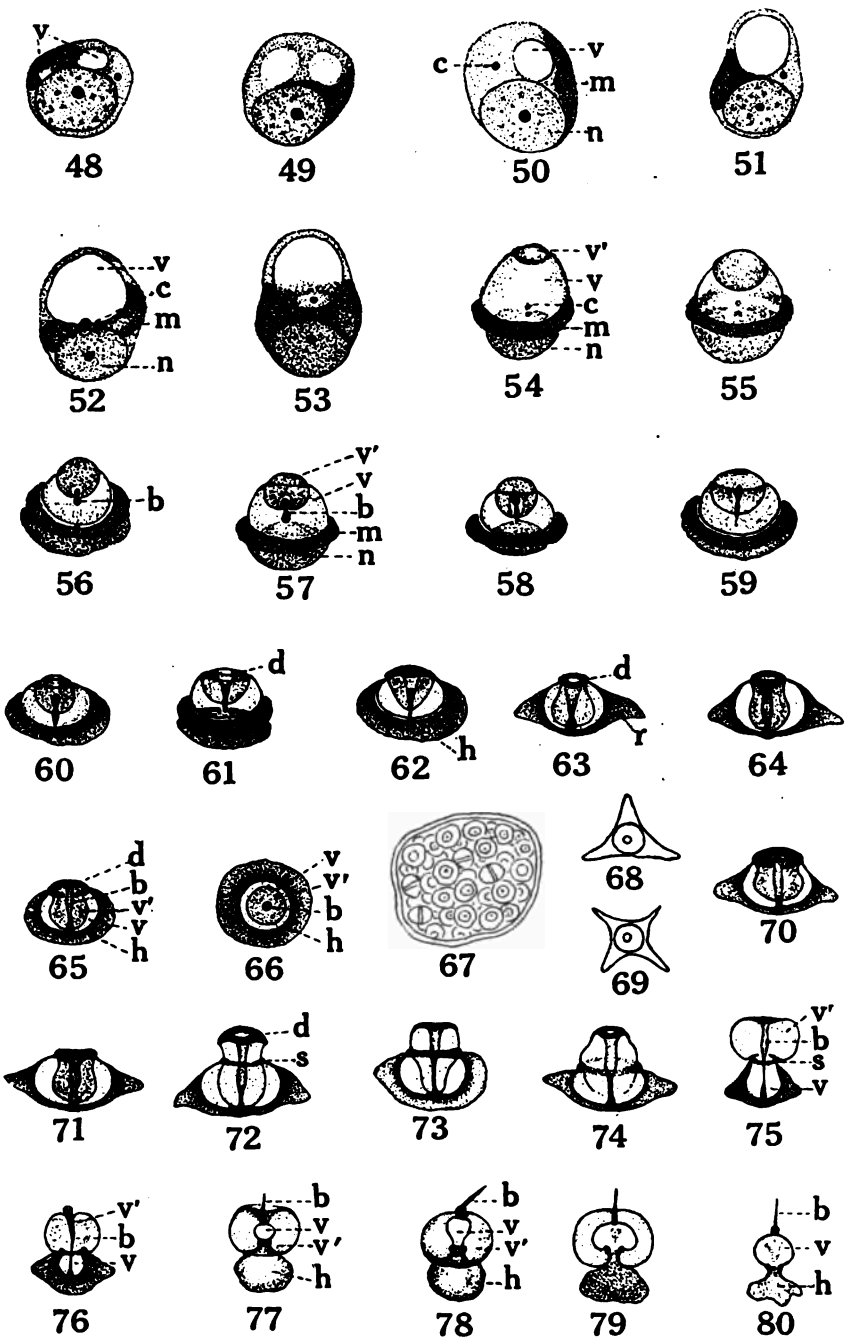

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OBSERVATIONS ON THE STRUCTURE OF PROTOPLASM BY AID OF MICRODISSECTION.

WILLIAM SEIFRIZ.

TECHNIQUE.

The introduction of the Barber pipette holder brought into use an exceedingly ingenous technique which promises to open up a great field of research, in which not only many of the results got from fixed material can be confirmed or refuted, but in which observations on the structure and behavior of living protoplasm can be made with an accuracy and certainty not otherwise possible. This instrument, originally designed to hold miniature pipettes for isolation and injection work, is equally well adapted for the manipulation of glass dissection needles. Each needle is held in a three-movement clamp and extends into a small moist chamber placed on the stage of the microscope. The vertical needle-tips project up into a hanging drop in which the material to be dissected is suspended.

A description of this instrument and of the technique connected with it was first published by Barber (1914). Chambers (1915) has given a brief account of its use, and later (1917*c*) published a full description of the instrument and of the ways of making micro-needles.[1]

LITERATURE.

The literature published on microdissection studies is limited practically to the articles of G. L. Kite and Robert Chambers. Both of these men employed a Barber instrument essentially identical with that used by the writer. A list of their publications is appended to this paper.

TERMINOLOGY.

A serious difficulty encountered by the investigator in this field is the limited vocabulary which is at his disposal for the

[1] It is of great importance in making sharp needles to have a minute flame. A very satisfactory micro-burner can be made—as was suggested to the writer by Dr. E. E. Free—from the smallest size (no. 27) hypodermic injection needle by snipping off the bevelled end with sharp shears.

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definition and description of the phenomena observed. We are little better off in choice of words than were the early microscopists whose knowledge of protoplasm was fully indicated by such adjectives as viscous, elastic, hyaline, etc.

In the possible range in consistency between the two extreme conditions of a colloid, *i. e.*, extreme liquidity and the solid state, the writer will distinguish ten degrees of viscosity, namely—watery, very liquid, liquid, slightly viscous, rather viscous, decidedly viscous, very viscous, extremely viscous, gel, and rigid gel—and has employed these expressions, for want of better ones, to describe the comparative viscosity of protoplasm. Their use will eliminate, in part, the vagueness from such general terms as liquid and viscous.

Protoplasm, which includes "all the living components of the cell-body" (Strasburger, 1891, p. 13), possesses many lifeless inclusions which materially affect its consistency, and they must be taken into consideration when the viscosity of protoplasm is discussed. As hyaloplasm is usually restricted to the hyaline border of non-granular plasma, the term matrix is used to indicate the translucent and more homogeneous fluid in which the various included granules are suspended.

Protoplasm, as such, frequently possesses a consistency different from that of its matrix. The latter may be watery yet the density of the protoplasm in toto markedly greater. It is, therefore, important to keep clear the distinction between the protoplasm as a whole and the matrix in which the various inclusions are imbedded.

MATERIAL.

The experimentation leading to the following results was carried on in the Harpswell Laboratory at South Harpswell, Maine. The writer wishes to thank the director, Dr. J. S. Kingsley, for the use of a room in the laboratory and for many other privileges enjoyed there.

The chief problem for the botanical microdissectionist is the obtaining of material delicate enough to permit dissection by fine glass needles. Even the wall of the frail alga *Spirogyra* can be entered only by the sharpest and most substantial needle that can be made. All in all, the most satisfactory objects for both zoö-

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logical and botanical workers are the ova of marine organisms, although the myxomycetes are in some respects still better, for we have in them the largest masses of pure protoplasm known.

The observations here published were made upon myxomycetes, pollen-tubes, the oögonia, ova, and embryos of *Fucus*, and the ova of *Echinarachnius*.

OBSERVATIONS.

MYXOMYCETES.

Plasmodia of *Ceratiomyxa*, *Badhamia*, *Arcyria*, *Cribraria* and *Fuligo* were studied.

The protoplasmic density of myxomycetes is such that a needle traverses the plasma as through water, although it exhibits a slightly viscid property, for inclusions are pushed ahead of and to the side of a needle before they actually come in contact with it. However, if the point of a needle is broken off, making of it a minute pipette, cytoplasm and small inclusions rush into the opening with great rapidity and from quite a distance.

The vegetative plasmodium of a myxomycete is of very liquid consistency and remains so no matter how thin the film or filament of streaming plasma may be. If the plasmodium is not active a film or isolated globule of its protoplasm gels very rapidly. The consistency becomes so dense that a moving needle leaves a permanent furrow.

The old appellation "naked protoplasm," much used in reference to the slime-moulds, is in important respects a misleading one. The surface layer of a plasmodium is a definite morphological structure. The membrane is very extensile, slowly contractile, and surprisingly tenacious for so delicate a layer. This superficial layer can be isolated and held by one needle while stretched to several times its length by the other.

Within the plasmodium are definitely bounded smaller masses of protoplasm, which are apparently normal and in every respect identical with the surrounding plasma bulk. The origin of these smaller included protoplasmic masses was not observed, but their presence is common and they are readily distinguished from oil or other liquid inclusions. Several of these globules were isolated and dissected. Their limiting membrane is exceedingly sensitive,

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breaking before a perceptible indentation can be noted. Further dissection gave convincing evidence that this membrane is a morphologically differentiated layer. On breaking a globule some of the liquid protoplasm escaped. The pellicle of the ruptured mass stood out prominently and could be handled with a needle as one would handle a hair. It was of no appreciable thickness, yet quite rigid, though easily bent with no indication of being soft or glutinous. It had, therefore, during dissection, undergone a change—the normal, and extremely sensitive membrane had become a tough rigid gel. Such behavior well supports our interpretation of the plasmodium pellicle—not a surface-tension membrane nor a secreted wall, but a bounding layer of denser protoplasm. The plasmodial membrane is, then, a gel of appreciable thickness, tenacious, very extensile, contractile, and glutinous.

Next in importance to the possession of a definite morphological membrane is the capacity for forming one. The evidence which dissection work on myxomycetes presents on this phenomenon is, briefly, that the membrane is instantly and repeatedly reformed when ruptured by a needle, provided the protoplasm is normal. This is true whether the dissection be performed on a dry cover or in water; that is, the capacity is unchanged whether air or water is the surrounding medium. If gelation has set in the capacity for membrane formation is lost, although it may persist surprisingly long.

The living substance of slime-moulds is non-miscible in water.

POLLEN TUBES.

Of the many pollen grains experimented with those of the large blue flag (*Iris versicolor*) were the most satisfactory. The grains are large and germinate readily in almost any per cent. of sugar solution. The pollen of the beach pea (*Lathyrus maritimus*) is a fair substitute.

Repeated irritation of a pollen tube puts an end to protoplasmic streaming, although in some instances streaming may continue even after a tube has been punctured, and a large amount of its contents lost. Streaming, however, is not accelerated by irritation, nor is there any indication of a rush of protoplasm to

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wounded regions. The active protoplasm of a pollen tube is of very liquid consistency. On ejection it gels, though it does so slowly. Brownian movement sets in shortly after.

Ejected masses of protoplasm from pollen tubes develop membranes immediately on being freed. The capacity for membrane formation persists one to two minutes. Within three minutes the escaped protoplasm has become quite viscous. The membrane formed is surprisingly tough. Fragments of it can be dragged into the escaped plasma mass.[1]

Escaping protoplasm shows no sign of miscibility. Isolated groups of inclusions exhibiting Brownian movement, after ejection, were carefully observed and in every instance the cytoplasmic matrix was distinctly visible.

FUCUS.

The eggs of *Fucus* develop from divisions of the contents of oögonia which arise from single superficial cells of the wall of conceptacles that cover the fruiting branches. If these oögonia are teased out at a very early age they can be entered by a sharp needle. Very soon, however, the outer wall (exochiton) becomes too hard to be penetrated.

Oögonia.

In consistency the protoplasm of young uninucleate oögonia is very liquid. The wall is thick (2–4 microns), tough, and highly resilient. Slightly older oögonia, but still uninucleate, also possess very liquid protoplasm. Stages in development between young uninucleate oögonia and almost mature oögonia can not be observed, as the tough outer wall does not permit of dissection.

Immature Ova.

The contents of nearly mature oögonia can be squeezed out of the heavy exochiton by crushing the conceptacle with tweezers.

[1] It was the intention of the writer to investigate the structure of the vacuole, of which so many are produced in the growing pollen tube. Freed vacuoles hold their shape even where exposed around the border of the plasma mass, apparently free of surrounding cytoplasm. When punctured the vacuoles collapse immediately. This behavior suggests that there is an enclosing membrane, and thus that the vacuole is apparently not really a vacuole but a sac. (De Vries, 1885, p. 467.)

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Unripe eggs obtained in this manner are still held together by a jelly mass. The earliest stages so obtained were of oögonia in which division was complete but the eight eggs still of pentagonal outline in profile and closely appressed.

The protoplasm is of liquid consistency but shows marked signs of an increased viscosity over that of young oögonia. It tolerates a great amount of ill-treatment without showing any signs of injury. After half an hour of dissection there was no indication of gelation, the viscosity of the protoplasm remaining the same. Brownian movement, that unfailing criterion of degeneration, was not seen.

Slightly older oögonia in which the eggs, though still closely appressed, have rounded up somewhat, show further increase in protoplasmic consistency to the slightly viscous stage.

The separating membranes of these closely appressed masses of protoplasm are exceedingly delicate and of inappreciable thickness.

The capacity for membrane formation is complete. A remarkable property of the protoplasm of a young *Fucus* ovum is the rapidity with which it is enclosed by a wall after a needle has severed the egg. The very elastic glutinous membrane, ordinarily barely visible, is sufficiently pliable to be made of appreciable thickness when pressure is applied laterally with a tendency to compress.

The above described behavior of the ripening *Fucus* egg leaves no doubt that the plasma is a non-miscible fluid.

The stage of development of the eggs just described must be borne in mind. These ova, of slightly viscous consistency and enclosed by a delicate membrane, are nearing the completion of their development. They are still closely appressed within the oögonium, with several hours intervening before they would have been discharged as ripe eggs with gelatinous contents and a thick, hyaline wall.

The protoplasm of eggs of more advanced oögonia is of greater density, namely, rather viscous. It flows readily, but slowly.

The last stage in the development of the eggs before their discharge is especially noticeable because of an increase in thickness of wall. The enclosing layer is now one half of a micron

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thick. Notwithstanding the possession of a heavier wall these eggs can be dissected with as much ease, ending in the same results as those less mature ova having but a thin pellicle as covering. Droplets can be pinched off, the eggs torn from within outwards, or rapidly severed by a needle, without any indication of escaping protoplasm. The severed parts in every case instantly round up into droplets with walls apparently identical with the parent wall. This capacity to form a wall instantly at a ruptured point through conversion of the cytoplasm into a semi-rigid gel is not influenced by relatively great changes in the concentration of the surrounding medium, for it is as pronounced in very saline water as in normal sea-water.

There is no evidence of miscibility on the part of the protoplasm.

Fully Mature Ova.

The protoplasm of mature, normally discharged eggs is decidedly viscous, noticeably more so than that of well-developed unripe eggs. Thus has the highest degree of viscosity been reached in the development of the ovum, the transition taking place during the last periods of growth.

The wall likewise has undergone a marked change and become a hyaline, rigid gel, 0.8–1.2 microns thick, still very pliable and extensile, slightly contractile and exceedingly adhesive.

The wall of the mature normal *Fucus* ovum is capable of constant repair and this capacity often persists to the very last in a dying egg. Not until gelation of the protoplasm is well advanced does a rupture of the wall fail to be closed by a rapid conversion of the plasma matrix into a rigid gel. The capacity for wall formation is, then, one of the last essential properties of the living substance to be lost.

In all stages of its development before its normal discharge the egg shows no injury in consequence of dissection. The mature egg, on the contrary, is very sensitive to dissection, although its behavior is extremely variable. Every precaution was taken to prevent a misinterpretation of results due to observation of degenerated protoplasm. Such precaution was found to be more necessary with the ova of plants than with those of animals. To determine that the ripe eggs used for dissection

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were normal, part of each lot used was kept and tested for fertility.

The response to dissection of ripe unfertilized ova may vary from immediate disintegration to the toleration of two severances. Many eggs are very easily pinched in half by two needles rapidly approaching from opposite sides. The halves frequently round up into perfect spheres which can be again severed to form smaller protoplasmic droplets. While some eggs will show but slight if any increase in viscosity after many minutes of slow movement of the needles, others will suffer little or no dissection before gelating or completely disintegrating. Mere puncturing of the egg will often cause gelation. Thus does the ripe egg exhibit in one instance great sensitiveness and in another apparent indifference to stimulus.

This variability is carried even to parts of the same egg. One half of a severed ovum may gelate immediately without forming a wall over the torn surface, while the other half tolerates still another severance before gelating and thus losing the capacity for wall-formation. The presence of the nucleus in one half of the egg may be responsible for this difference in behavior (Townsend, 1897), although frequently both halves develop enclosing walls.

The protoplasm of the living ovum is at all times non-miscible in sea-water. This is to be expected if the capacity for wall-formation persists as long as the protoplasm is alive. In no instance of eggs examined in the various stages of ripening, while mature, and in the brief period following fertilization when the wall was still penetrable, was the plasma found to pour out and mix with the surrounding water.

The Unicellular Embryo.

Fertilization in *Fucus* is readily accomplished if active sperm are placed on a slide with mature ova.

Half an hour after application of the sperm the protoplasm of the fertilized ovum is found to be still quite viscous. A little later it becomes more liquid, for it readily oozes out of a puncture. Further development of the unicellular embryo shows a continued decrease in protoplasmic density.

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The wall immediately after fertilization shows no increase in thickness and is still very pliable, extensile, and contractile. A little later it stiffens up until no longer elastic, though slightly resilient and pliable in so far that permanent indentations can be made. It can not now be severed by two approaching needles. In those few instances where the tough wall was punctured, and the protoplasm forced out, the ejected mass immediately developed a membrane. This plasma membrane is exceedingly thin, and quite elastic. It is interesting to note that the freed protoplasm here forms a delicate pellicle—not the tough wall of the one-celled embryo from which it came, nor the substantial though glutinous egg-wall which it would have developed in repairing a tear before fertilization.

At all times, from the moment of fertilization until dissection is no longer possible, the embryonic protoplasm is non-miscible. The ejected plasma shows no tendency to mix with the surrounding sea-water until after gelating, *i. e.*, after death.

The Multicellular Embryo.

As the two-celled embryo stage is neared the former egg wall has lost all of its former elasticity and glutinosity. It behaves under pressure like a ball of thin celluloid, resisting light pressure and returning to its former shape, unless forced beyond a certain point, when depressions made are retained. The two-celled embryo is punctured with great difficulty. The wall of the four-celled embryo is a very substantial affair, two microns in thickness, highly resilient and impenetrable to the sharpest needle.

The Degenerate Egg.

The behavior of the over-ripe ovum is extremely interesting and instructive in its bearing on the mechanism of the egg contents. As already stated, an ovum ultimately gelates and usually disintegrates. A gelated egg may be torn apart with great ease. If hardening has progressed far enough the egg may be cut in pieces as one would cut butter. If, on the other hand, the contents are not too solid the matrix may be readily drawn out into a long invisible thread. It is this gelated cytoplasmic matrix which is water-miscible.

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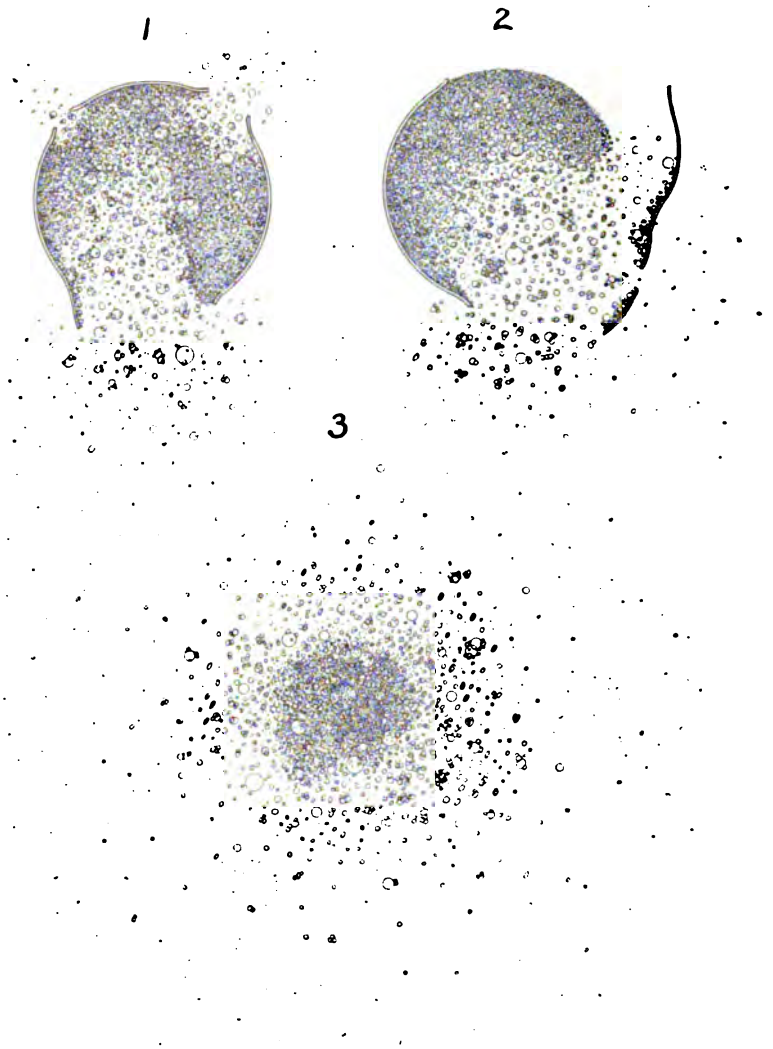

DESCRIPTION OF DRAWINGS.

FIG. 1. A disintegrating *Fucus* ovum in which smaller craters have burst forth subsequently to several seconds of activity on the part of the larger crater.

FIG. 2. A degenerate *Fucus* ovum in which discarded fragments of the wall are seen, and in which dissemination of granules is very active in one region while non-existent in another.

FIG. 3. The wild and rapid scattering of the inclusions of an exploded *Fucus* egg.

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The disintegration of a degenerate egg is accomplished by the matrix going into solution with the sea-water. This may take place with such rapidity as to suggest a miniature explosion of the egg; or may be delayed and then it usually proceeds slowly, either in spasmodic periods of dissolution, or continuously until the entire granular contents has been disseminated. Often this is never completed. Where the disintegration is instantaneous (Fig. 3) it takes place immediately on the over-ripe egg coming in contact with a needle, or by the egg itself before actual dissection can be got under way. The miscibility of the matrix may be general (Fig. 3), or localized (Fig. 1). Where dissolution takes place without dissection some internal pressure, evidently osmotic, brings about a rupture. But it is difficult to see why such a force does not greatly expand so pliable a wall before breaking it. This is not the case. Furthermore, where disintegration has been instantaneous wall fragments are not to be found. When the process is gradual bits of it are only occasionally seen (Fig. 2). There is some reason for believing that the pliable, glutinous wall itself undergoes a change during degeneration of the protoplasm. If it does not go into solution it probably becomes a soft, inelastic gel.

A distributed, internal, osmotic pressure would be relieved once the egg-wall had broken at any one point, and one would not expect further eruptions. Quite the contrary is true. There may be a rather general dissemination of granules from one region for several seconds, time enough to relieve any pressure within, and subsequently, other craters break forth as shown in Fig. 1. These are localized centers of activity. That scattered regions of the egg contents are in different physiological states is further evident from the fact that certain localities may from the very outset remain inactive, never taking part in the general dissolution of the protoplasm, such as the exposed upper surface in Fig. 2.

The force responsible for a wild scattering of the inclusions in an ovum is a surface-tension one, due to an extraordinarily rapid miscibility of the degenerate matrix in the surrounding medium of sea-water.

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ECHINARACHNIUS.

Microdissection work on the egg of the sand-dollar was undertaken for the purpose of comparison with the behavior of the plant ovum.

The difference in structure and behavior of *Fucus* and *Echinarachnius* ova before fertilization is not great. With fertilization two striking dissimilarities originate. There is in *Echinarachnius* an increase in viscosity of the protoplasm after fertilization. The path of a needle closes very slowly. Furthermore, there is very little change in the character of the egg wall. The wall of the young embryo is of no greater thickness than that of the ovum and but slightly more resistant.

The *Echinarachnius* egg occasionally tolerates a very great amount of dissection before completely deteriorating. At times, the capacity for wall formation persists in a region of still liquid plasma even after other regions of the egg have become an exceedingly viscous mass. This illustrates further the great diversity in the behavior of the protoplasm of eggs existing under apparently identical environments, and the marked difference in physiological condition of different regions of the same cell.

During the dissection of more than 20 eggs of the sand-dollar but one instance of miscibility was observed, and this was the rapid dissemination of granules due to the going into solution of the matrix of a gelated, that is, a degenerating ovum.

DISCUSSION.

In spite of the variety of objects studied and the differences in behavior of very similar material there are certain definite properties which characterize all the protoplasm here observed.

Streaming protoplasm is of a very liquid consistency. The same is true of young, actively growing plasma such as that found in the developing oögonium and the embryo of *Fucus*. The increase in consistency of the *Echinarachnius* ovum following the entrance of the sperm is not in harmony with this fact, nor is it what one would expect; for the liquid condition of protoplasm enables the elaborate chemical reactions of an active cell to take place. In the ripe egg, awaiting fertilization, metabolism is reduced to a minimum, so that a liquid state is not needed as it

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is for the complex chemical activities and interchange of substances which go on in the developing egg and growing embryo. In accordance with this is the fact that normal passive protoplasm in mature eggs of *Fucus* and *Echinarachnius* ova is quite viscous. (In ripe and resting seeds the protoplasm becomes almost solid.)

There is no reason for believing that any living protoplasm is naked. The surface membrane of protoplasm is at all times a definite morphological structure. The capacity to form such a pellicle is one of the characteristic properties of the living substance and is retained to a very late stage in dying protoplasm.

The membrane formed in repairing a tear is of the same character as the original. This is not true in those instances where the wall is of cellulose, as in pollen-tubes and plant embryos; for the enclosing surface layer of protoplasm is but a transformed portion of the living substance, the result of an immediate conversion of liquid plasma into a rigid gel of greater molar concentration. (Pfeffer, 1891, p. 194). It is worthy of note that protoplasm possesses not only the capacity to form a membrane but that kind of a membrane characteristic of a particular organism or of a particular stage of development. This suggests that the process is to some degree controlled from within.

That contact with a *certain* medium is not necessary for membrane formation is evidenced from the fact that streaming myxomycetes form membranes whether dissected on a dry coverslip or in a hanging drop, and that the pellicle of the *Fucus* ovum is repaired whether it is torn open in the jelly-mass of the oögonium or in sea-water. Thus does it appear that contact with some medium is not a prerequisite to membrane formation, the lack of a membrane being the stimulus. Membranes are formed on any free protoplasmic surface. The general belief is that this is a purely physical process, and owes its origin to surface forces. It is somewhat disturbing to this strictly mechanistic conception that the capacity for membrane formation ends with death. (Pfeffer (1877) has shown experimentally that a membrane may be formed after death. Chemistry could give us many such instances. In neither case is the membrane a natural one.) Surface forces certainly come into play, but the capacity for membrane formation, and beyond doubt the factor determining

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the kind of membrane formed, is dependent upon the physiological state of the protoplasm, which, in a sense, means that the ultimate control lies within the cell; though, of course, not beyond the purely physical and chemical properties of the living substance.

The prevailing idea of a cell wall is that it is a dead structure and, therefore, incapable of change or response to environment. This is probably true of the cellulose walls of older cells, but not of the distensible, glutinous membranes and egg-walls here discussed nor of the cell-walls of meristematic tissues in general. The suggestion that the wall of a degenerating ovum may and does undergo, with the egg-protoplasm, a change which permits either its going into solution or its ready rupture by internal osmotic pressure, is not without experimental support. Protoplasmic membranes are an intimate part of the living substance and susceptible to the same changes in environment. (Bayliss, 1916, p. 115.)

Normal protoplasm, in all cases studied by the writer, does not mix with water. This declaration is contrary to that of Chambers (1917*a*, p. 2). If the decision is to rest on evidence gained solely from microdissection then miscibility of the protoplasm is a consequence of degeneration. Chambers's statement, however, rests not only on microdissection but on the introduction of water into the cell by the mercury injection method as well. Miscibility precludes the presence of a membrane. Normal protoplasm is always capable of membrane formation. Therefore, normal protoplasm can at no time be miscible. The dissection of a great many eggs supports this conclusion.

To the highest powers of the microscope, with ordinary illumination, protoplasm is a homogeneous, structureless solution. Its colloidal nature becomes evident on using the dark ground illuminator. Protoplasm is probably an emulsoid hydrosol, *i. e.*, a colloid in which both phases are liquid, one of them—the dispersion medium—being water.

Any definite statement on the ultimate structure of protoplasm must be expressed in physicochemical terms, and based upon observations made with the ultra-microscope. The behavior of protoplasm under dissection, however, throws considerable light on the gross structure of the cell contents. In dis-

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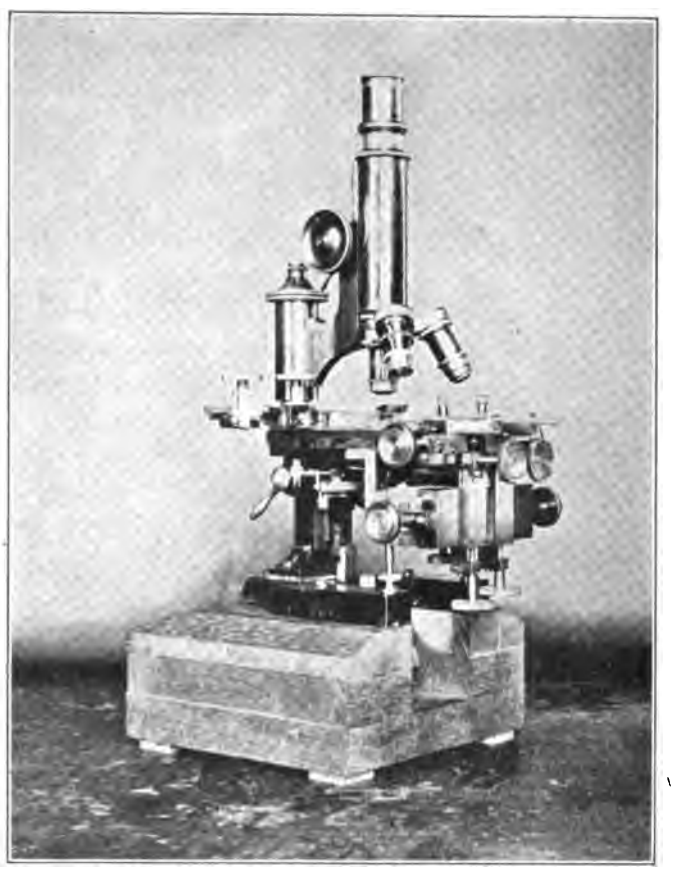
tinguishing between gross and ultimate structure the writer has in mind, under the former, a haphazard arrangement of the minute subdivisions or centers of activity, of the living substance which are at any one moment in differing physiological states;



DESCRIPTION OF PHOTOGRAPH.

A two-needle Barber pipette holder attached to a Leitz microscope.

while an ultimate structure connotes a definite arrangement of molecules or of the infinitesimal particles seen through the ultramicroscope—the microns or inferred amicrons of which all colloidal systems are composed.

The most remarkable feature of life within a cell is the coexis-

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tence of a great number of reactions, constantly going on without one interfering with the other. This demands the division of the contents into innumerable chambers or centers of activity. Thus does the protoplasm possess, in a sense, structure. The presence of these miniature laboratories is well illustrated by the behavior of disintegrating ova in which the dissemination of the inclusions is at times in certain regions extremely rapid, and in others non-existent (Figs. 1 and 2). The difference in the physiological state of the protoplasm at any one moment is responsible for the variety of behavior of eggs from the same oögonium and of parts of the same ovum. This difference is to be expected if protoplasmic activity takes place in pulsations, which are neither synchronous among the eight eggs of one oögonium nor rythmic in any single ovum. The cell is, then, a laboratory in which many different chemical reactions are constantly going on, kept free from one another by boundaries of some kind. (Hofmeister, 1901.)

Structure in protoplasm is secondary to activity. Upon the chemical nature of the substances does the life of the cell depend rather than on their arrangement. What physical structures may exist are of a transitory nature. This interpretation does not preclude the all-important organization upon which the continuance of these activities depends.

SUMMARY.

1. Protoplasm is an emulsion colloid normally in the sol state.

2. The density of protoplasm varies from the very liquid state of young *Fucus* oögonia and embryos and of streaming protoplasm in myxomycetes and pollen tubes, to the quite viscous condition found in mature and resting eggs of marine organisms.

3. There is a rapid increase in viscosity of the *Fucus* egg during the last stages of its ripening, which is, on fertilization, followed by a return to the liquid consistency characteristic of active, growing proptolasm.

4. The plasma membrane is a definite morphological structure, constantly and repeatedly capable of repair through the conversion of the fluid protoplasm into a hyaline layer of greater molar concentration. This film of gel is exceedingly elastic, pliable and glutinous.

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5. The surface layer, like the interior cytoplasm, seems to be capable of alteration with changes in environment.

6. The capacity for membrane-formation is one of the last essential properties of the living substance to be lost. It is lost only at death.

7. The kind of membrane formed is apparently identical with the parent membrane (except in the case of escaped protoplasm from cells which possess a cellulose wall).

8. The formation of a membrane is probably a purely physical process, but is dependent upon the physiological condition of the protoplasm. It is not dependent upon the surrounding medium.

9. The amount of physical disturbance that protoplasm can be subjected to before showing signs of injury varies from that of the immature *Fucus* ovum, where it is exceedingly great, to that of the ripe egg where it is very slight, often no more than a touch sufficing to cause disorganization.

10. Gelation of the plasma always takes place in time and is hurried by dissection. It is accompanied by degeneration.

11. Normal protoplasm is at all times non-miscible in water. Miscibility of the plasma is an unfailing criterion of degeneration.

12. Dissolution of the *Fucus* ovum is the result of the disorganized cytoplasmic matrix going into solution with the surrounding water. This mixing may take place with the rapidity of an explosion, or slowly, and then either continuously or spasmodically.

13. The disintegration of the egg plasm is frequently localized, in that certain regions of the contents continue dissemination of the granules from the beginning, while others join in later, and still others never take part. This indicates a gross structure of the egg plasm, *i. e.*, the protoplasm is composed of many centers of activity in which different chemical reactions take place separated by protective partitions.

14. That any definite and permanent arrangement of the colloidal particles exists seems unlikely. Whatever structure, gross or ultimate, protoplasm may possess is secondary to chemical activity upon which the life of the organism depends.

The writer wishes to thank Professor Duncan S. Johnson for suggesting this study and for assistance during its progress.

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To Dr. Robert Chambers the writer is indebted for helpful suggestions, and to Dr. E. V. Cowdry for the use of his Barber instrument.

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FURTHER STUDIES ON THE PRODUCTION OF FUNCTIONAL AND RUDIMENTARY SPERMATOZOA IN ROTIFERS.[1]

D. D. WHITNEY.

In a recent paper some general observations were recorded in regard to the dimorphic spermatozoa found in the parthenogenetically developed males of nine species of rotifers. Some of the stages in the development of the spermatozoa from the cells of the last spermatocyte-divisions in *Brachionus mulleri* were described and figured. No studies, however, were made on the nuclear material of the spermatozoa either to determine whether it was present in both kinds of spermatozoa or to determine in what part of the spermatozoa it was located.

Additional studies now have been completed in an attempt to observe these points. These studies have been made in considerable detail on the marine species, *Brachionus mulleri*, and in lesser detail on *Hydatina senta*, *Brachionus amphiceros* and *Diaschiza sterea*. As the earlier paper had some of the details of the formation of the spermatozoa of *Brachionus mulleri* and as this rotifer is one of the most convenient forms to maintain in the laboratory and also a very convenient one from which to obtain immature males the majority of the observations were made on material from this species.

Several killing and fixing fluids and stains were tried but Zenker's fluid followed by Delafield's hematoxylin proved to be the most satisfactory method for making clear mounts of the entire cells. The living males were crushed under a cover glass in culture water and favorable cells were selected under the microscope. The killing fluid was allowed to run under the cover glass and to remain from 10-20 minutes. Later the killing fluid was drawn out by filter paper and distilled water allowed to take its place. After the distilled water had been changed several times from under the cover glass Delafield's hematoxylin

[1] Studies from the Zoölogical Laboratory, The University of Nebraska, No. 119.

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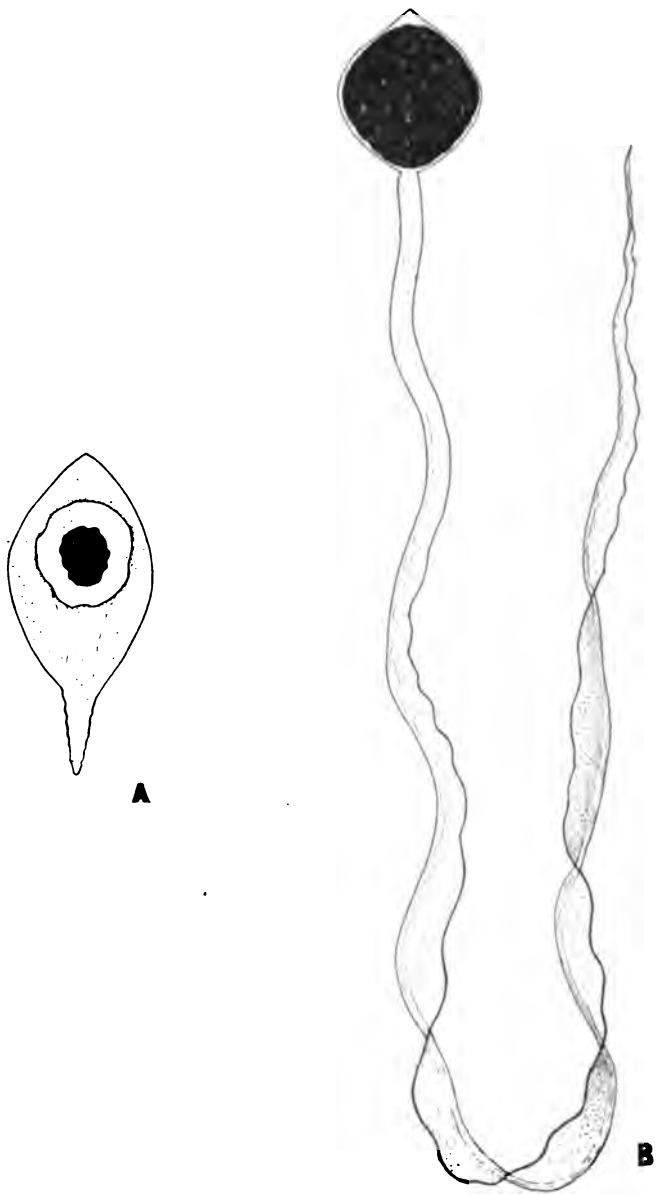

FIG. 1. *Brachionus mulleri.* *A*, normal spermatid showing the nucleus, chromatin, and the small tail; *B*, mature and normal spermatozoön showing the large chromatin mass in the head and the long vibratile tail with the undulating membrane. (Drawn by measurements from living and stained specimens with 1/12 oil immersion objective and a number 2 ocular.)

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(diluted 1:1) was allowed to run under and to stain the cells for about two minutes. This was then washed out with tap water. All of these processes were carried on under the microscope in order to make certain that no mistakes of indentification of these cells should be made after the slides were finished. Although this is a tedious method it has the advantage of being perfectly reliable and furnishing accurate data concerning which there can be no doubts. Many other methods were tried but none were found to be as practical and as certain as this one.

The nucleus was first indentified in an early stage of the normal spermatid (Fig. 1, *A*) which later would have developed into the large motile spermatozoon (Fig. 1, *B*).

The degenerate sperm cells from the same male individual were examined next and each was found to contain a nucleus with chromatin material in it. This stage is shown in Fig. 2, *A*. This fact is of considerable interest because it demonstrates that in the division that forms the secondary spermatocytes there is evidently a division of the chromatin material and consequently all the secondary spermatocytes contain some of this chromatin material. One half of these secondary spermatocytes divide and form the normal spermatids but the remaining half of the secondary spermatocytes do not divide again but develop directly into the degernerate spermatozoa. The early stage of these degenerate spermatocyte cells are smaller in size than the normal cells of the same age but the nucleus is only slightly smaller than the nucleus contained in the normal spermatid cell as can be seen by comparing Figs. 2, *A* and 1, *A*.

The later development of each of these two kinds of cells is very different. In the normal cell the nucleus grows larger and larger until in its final stage it is several times its former size. From one end of the cell an outgrowth appears which grows longer and longer and finally becomes vibratile. This is the motile tail of the sperm cell. It is very long and large and has an undulating membrane along the greater portion of the dorsal side. Fig. 1, *B*, shows the entire matured spermatozoön containing the large mass of chromatin material in the head.

The development of the smaller and degenerate spermatocyte cells into the complete rudimentary spermatozoa is strikingly

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different from the development of the normal spermatid cells. After the early stage is passed the chromatin material increases in bulk very slightly and becomes composed of coarse granules

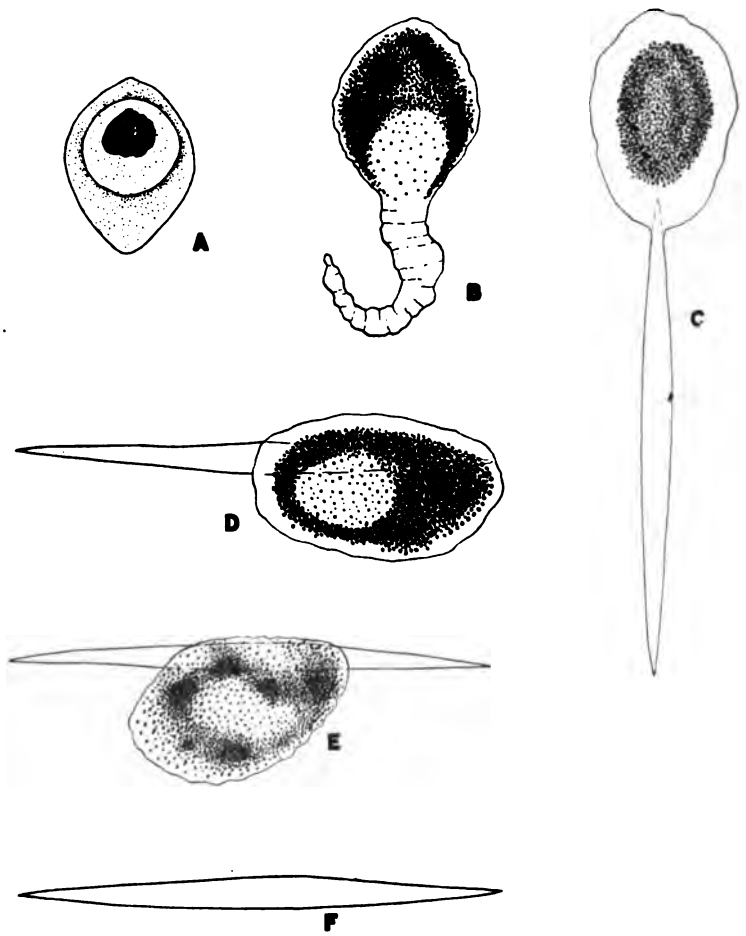

FIG. 2. *Brachionus mulleri*. *A*, early stage in the development of the rudimentary spermatozoön showing the chromatin in the nucleus. This cell is from the same male as the normal spermatid of Fig. 1, *A*, and is of the same age. *B*, a later stage showing the tail wrinkled and distorted by the fixing fluid; *C*, last stage showing the stiff and rigid tail attached to the head which contains the chromatin; *D*, the stiff tail forced through the chromatin to the opposite side of the head by pressure under the cover glass; *E*, the stiff tail forced through the opposite wall of the head by pressure under the cover glass; *F*, the stiff tail detached from the head. (Drawn to same scale as Fig. 1.)

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loosely arranged and persists in this form throughout the remainder of the development of the cell. From one end of the cell a process extends which is somewhat flexible in its earlier development. Fig. 2, *B*, shows such an early stage in which the tail-like process has become curved and wrinkled after treatment with Zenker's fluid. Later this tail-like process becomes very rigid and stiff. So stiff that when the immature males are crushed this process by chance may be driven through the nuclear material to the opposite side of the cell as shown in Fig. 2, *C*, or in some instances it was even driven through the opposite wall of the cell as shown in Fig. 2, *D*. Normally this stiff process seems to be attached to one end of the cell. Later, however, when it is fully formed it becomes detached from the cell. This separation of the two parts of the cell occurs in the testis either before the male hatches from the egg or very soon thereafter. Fig. 2, *C*, shows the two parts just prior to separation.

This stiff spindle-shaped part contains no chromatin material and probably is to be considered the rudimentary tail of the cell. It is immotile and much smaller than the tail of the motile spermatozoön. As it separates from the nucleated portion of the cell and is immotile one could naturally conclude that it is to be regarded as simply a degenerate or rudimentary tail which has no function. Whether the nucleated portion of the cell has any function is unknown but as it is also immotile it is also very probably functionless.

In the earlier studies the detached tails of the normal spermatozoa were erroneously considered to be the entire spermatozoa. When the males were crushed under a cover glass these tails were extruded and were able to move about in the water for a considerable length of time. When recently, however, these so-called spermatozoa were stained no nucleus or chromatin material could be found in any of them. As they are the functional spermatozoa it was realized that there must be a nucleus and chromatin material somewhere in them.

It was recalled that in previous observations some males had been seen to extrude a few of these motile bodies which had a large swelling on the anterior end. At that time these motile bodies were considered to be the immature spermatozoa. When

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more careful observations were made both upon such living cells and upon stained ones it was readily seen that these cells were not immature stages but were the mature stages of the spermatozoa. The heads are quite large in proportion to the diameter of the tail, Fig. 1, *B*, and are easily separated from the tails. In the testis of the male these spermatozoa are in clusters and in each cluster each spermatozoön is attached to some sort of a central tissue in the cluster. Normally in copulation with the young females a few of these spermatozoa become detached from the central tissue and are extruded into the body cavity of the female. When, however, the males are crushed under a cover glass nearly every head remains attached to this central tissue but the tails break off from the heads and are immediately extruded in a writhing mass. If the males are sufficiently crushed the mass of spermatozoa heads will also be extruded together with other cells of the broken-down tissues. If very young males are taken from the eggs and are crushed all of the immature sperm cells with the tails attached will be extruded. The ripe spermatozoa resemble very closely these immature spermatozoa except their heads are more rounded and the tails are somewhat longer.

The males of another rotifer, *Hydatina senta*, were crushed and the spermatozoa reëxamined. A few males shed both kinds of spermatozoa with the tails attached to the heads. These were stained and the heads of each kind were found to contain chromatin material similar to the heads of the spermatozoa of *Brachionus mulleri*. These are shown in Fig. 3. Twenty-five males were crushed and out of the many hundred spermatozoa ejected only about 6–8 entire spermatozoa of each kind with the tail attached to the head were found. *Hydatina senta* males are the most favorable material thus far examined in which to see the two kinds of complete spermatozoa in a mature male. In *Brachionus mulleri* these two kinds of complete spermatozoa could only be found in the unhatched and immature males.

The spermatozoa were examined from mature males of two additional species of rotifers, *Brachionus amphiceros*, and *Diaschiza sterea*. The entire normal spermatozoa with the tails attached to the heads were found in each species. Upon staining

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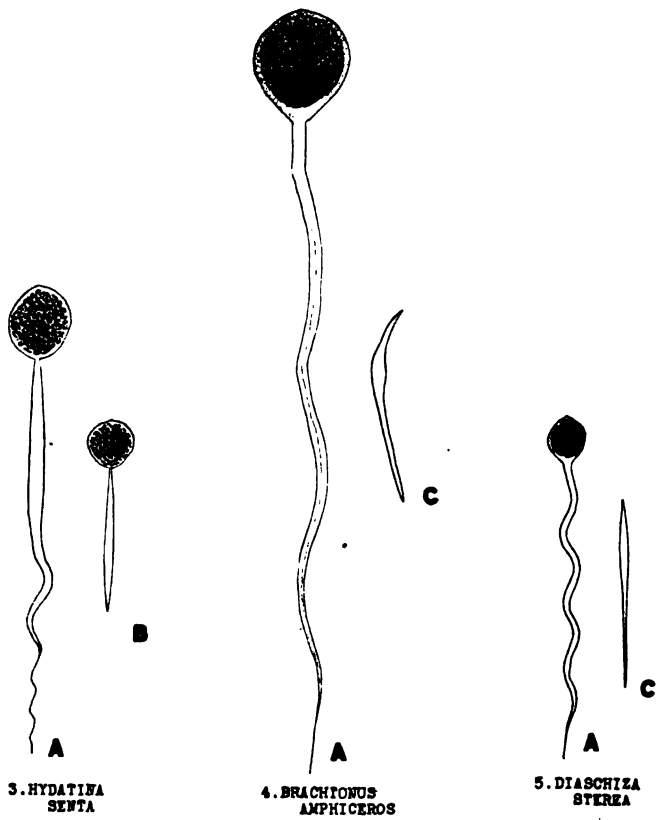
the heads showed their chromatin contents. Only the tails of the rudimentary spermatozoa were found and they showed no chromatin material in them when stained. Sketches of these spermatozoa and tails are shown in Figs. 4 and 5.



FIGS. 3–5. Dimorphic spermatozoa and tails of three other species of rotifers. *A*, larger normal and motile spermatozoa showing the head and the contained chromatin; *B*, smaller, rudimentary and non-motile spermatozoön showing the chromatin in the head; *C*, detached tails of rudimentary spermatozoa. (Drawn with 4 mm. objective and number 2 ocular.)

The spermatozoa of the other species of rotifers enumerated in the former paper have not been reëxamined because these forms are not available at this season of the year.

As the stained slides of the sectioned unlayed fertilized eggs of *Hydatina senta* were available for study they were examined to

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determine the size and condition of the head of the spermatozoön after it had entered the egg. The egg examined was sectioned while inside the body of the female and although it had the thick secretion around it which later would have shrunken to form the heavy external covering or shell it was readily sectioned at this

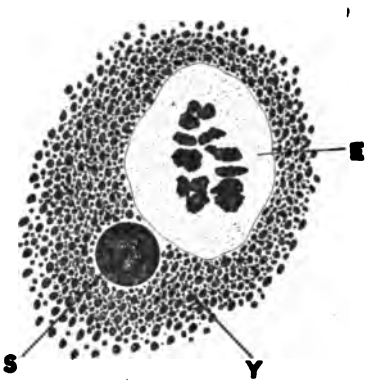

FIG. 6. Portion of a fertilized egg of *Hydatina senta*. *E*, egg nucleus containing chromosomes; *S*, sperm-head; *Y*, yolk granules of the egg. (Sperm-head and the outline of egg nucleus drawn to same scale as in Figs. 3–5. Yolk granules and chromosomes drawn freehand without measurements.)

early stage. The sperm head is seen near the nucleus of the egg (Fig. 6). It is about the size of the head of the normal spermatozoön as can be seen by comparing it with Fig. 3, but contains denser chromatin material and is perfectly round in form.

Dimorphic spermatozoa have been studied in considerable detail in some of the Mollusca and in some of the Lepidoptera by several workers. Among these Goldschmidt and Gatenby recently have come to the conclusion that the atypical or abnormal spermatozoa are wholly functionless and the latter is of the opinion that such spermatozoa have no significance in regard to sex regulation. According to their conclusion any spermatozoa and in some cases all spermatozoa of these forms studied may become degenerate if the male individual is in a certain physiological condition.

In the phylloxerans and in the rotifers the case is quite different. In each and every male a certain percentage of the sperm

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cells degenerate and a certain percentage of the sperm cells develop normally, thus giving a definite ratio of degenerate and normal sperm cells. In the phylloxerans this ratio has not been determined but in the rotifers there is one degenerate sperm cell to two normal sperm cells. As all the fertilized eggs in both phylloxerans and rotifers develop into female young it seems safe to conclude, as Morgan has already concluded, that the degenerate sperm cells are the male-determining ones and that the normal sperm cells are the female-determining ones.

Summary.

1. The normal and motile spermatozoa of *Brachionus mulleri*, *Brachionus amphiceros*, *Hydatina senta* and *Diaschiza sterea* possess large heads in which is located the chromatin material.

2. The motile bodies of seven other species of rotifers which were figured and considered as the normal spermatozoa in the former paper were probably only the tails of such normal spermatozoa.

3. The degenerate and immotile spermatozoa as seen in *Brachionus mulleri* and *Hydatina senta* also possess heads in which there is chromatin material.

4. The stiff processes, called rudimentary spermatozoa in the former paper, are now considered to be not the complete rudimentary spermatozoa but only the degenerate tails of these rudimentary spermatozoa.

5. The sperm head in the fertilized egg of *Hydatina senta* is about the same size as the head of the normal spermatozoön of this species.

6. The four species of rotifers enumerated above together with the seven other species described in the former paper constitute eleven species of rotifers in which both normal and rudimentary spermatozoa have been found to occur.

Zoölogical Laboratories,
The University of Nebraska,
Lincoln, Nebraska, January 5, 1918.

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BIOLOGICAL BULLETIN

EFFECT OF ENVIRONMENT UPON INHERITED CHARACTERS OF *HYDATINA SENTA*.

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INTRODUCTION.

Several years ago it was discovered (Shull, 1915) that two distinct parthenogenetic lines of the rotifer *Hydatina senta*, one from England, the other from Nebraska, differed in certain physiological (and perhaps structural) characters: (1) the English line laid smaller eggs than the Nebraska line; (2) the English line habitually laid a large percentage of its eggs attached to the surface film of the water, while the Nebraska line laid most of its eggs at the bottom or sides of the vessel; (3) the eggs of the English line required longer to develop than did those of the Nebraska line; (4) and when the rotifers were killed in Bouin's fluid the foot was seldom, or only slightly, retracted in the English line, but considerably retracted in the Nebraska line.

When crosses were effected between these two lines, the F_1 lines and F_2 lines were all indistinguishable from the English line in all the above-named characteristics. It seemed as if segregation and recombination had failed, and that in some way the four characters were rigidly associated one with another.

At first it was regarded as possible that the four characters were not really distinct, but were different manifestations of a single (physiological) character. That character might have been a greater permeability of the cells in one line than in the other. Thus, if the Nebraska line were more permeable to oxygen, the increased metabolism might make its eggs larger. For the same reason the eggs of the Nebraska line might develop in less time. In like manner it might be that the Nebraska rotifers, able to get the required amount of oxygen at the bottom

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of the dish where the oxygen in solution was less abundant, lived most of the time at the bottom and laid their eggs there; whereas the less permeable English rotifers were forced to swim to the surface where dissolved oxygen was presumably more abundant, and laid eggs at the surface film. And if the foot muscles of the Nebraska line were more permeable to the killing fluid than were those of the English line, the greater contraction of the muscles of the former might thereby be explained.

It was possible to test the correctness of the above assumptions, in part, by artifically altering the expression of the inherited characters through changes in the environment. A number of experiments were performed to this end. However, before they were completed, an F_3 generation was obtained in which the association of the four inherited characters was broken. In this and the succeeding generations each one of the four characters was separated at least once from the others with which it was associated in the original lines.

Thus was proven that the four characters were not merely different expressions of one character. The experiments designed to test their separateness or singleness were, therefore, not completed, and were not published. It has now become necessary, however, to refer to certain of the results, and they are here described in the incomplete form in which they were left. Along with them are several experiments on the viability of fertilized eggs, as affected by external conditions. These are of interest to the experimenter from a practical standpoint, and also in relation to popular ideas concerning the fertilized eggs of *Cladocera*.

Experiments.

Effect of oxygen upon the laying of eggs at surface and bottom of water.

On each of the dates named in Table I., approximately equal numbers of females of *Hydatina* were placed in two dishes. In one was placed water oxygenated by vigorously shaking it in an atmosphere having a high percentage of oxygen. The dish was then set, uncovered, under a bell jar in which was confined an atmosphere containing the same high percentage of oxygen as that with which the water was first saturated. Since the bell

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jar was being used daily for other experiments, the percentage of oxygen used was not always the same. From May 27 to June 22, and on July 8 (see Table I.), the atmosphere contained 40 per cent. of oxygen; on all other dates 60 per cent.

In the other of the two dishes was placed untreated water. This dish was also set under a jar sealed at the edges, to prevent excess of evaporation, but in ordinary air.

After 20 to 40 hours the dishes were removed and the number of eggs at the surface film and at the bottom counted. As shown in Table I., the eggs are much less abundant at the surface film in the presence of much oxygen than in air.

TABLE I.

Showing the Number of Eggs Laid at the Surface Film and at the Bottom of the Dish by Rotifers Placed in Air and in an Atmosphere Containing an Excess of Oxygen.

Date.	Air.		Excess of Oxygen.	
	Number of Eggs at Surface.	Number of Eggs at Bottom.	Number of Eggs at Surface.	Number of Eggs at Bottom.
May 27	12	5	1	15
29	37	48	16	55
June 3	22	22	16	27
22	2	42	0	25
28	62	20	19	49
29	8	25	7	29
30	43	85	18	121
July 1	38	19	17	27
2	40	33	34	39
3	45	6	14	41
4	42	36	23	76
6	12	5	8	22
8	23	10	12	22
Total	386	356	185	548
Percentage at surface	52.0		25.2	

Effect of oxygen upon the size of parthenogenetic eggs.

Eggs laid by rotifers in oxygenated water, and in untreated water, were obtained in the following manner. Three or four young females, due to begin egg laying in 6 to 12 hours, were placed in each of two watch glasses. One lot was immersed in water saturated with an atmosphere of which 40 per cent. or 60 per cent. was oxygen, and then set under a bell jar in a similar atmosphere. The other lot was placed in spring water, and the dish was set under a closed bell jar in air. After 24 hours the

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watch glasses were removed, and all the eggs that were in a horizontal position were measured by means of a screw micrometer eye-piece. The measurements are given in units of the scale. They are directly comparable with the measurement of eggs in my former paper (Shull, 1915) since all the measurements were made with the same microscope and with the same lenses.

The mean length, mean breadth, standard deviation, etc., of the two lots of eggs are given in Table II.

TABLE II.

A Comparison of the Eggs Laid by the Rotifer Hydatina Senta in Oxygenated Water and Untreated Water.

	In Oxygenated Water.	In Untreated Water.	Difference.
Number of eggs measured.....	100	100	0
Mean length of eggs.....	16.208 ± 0.028	16.098 ± 0.027	0.110 ± 0.039
Mean breadth of eggs.....	14.054 ± 0.021	14.021 ± 0.018	0.033 ± 0.028
Standard deviation of length...	0.409 ± 0.019	0.404 ± 0.019	0.005 ± 0.026
Standard deviation of breadth..	0.308 ± 0.015	0.266 ± 0.013	0.042 ± 0.019

The eggs in the oxygenated water were a trifle larger than those in the untreated water, though it can hardly be said that the difference is statistically significant. However, a difference that cannot be proven by statistical treatment to be significant is not necessarily insignificant. It seems to me not improbable that the difference in length is significant, but it is very small in comparison with the difference between English and Nebraska eggs described in my earlier publication (*op. cit.*).

Effect of oxygen upon the time of development of parthenogenetic eggs.

A number of egg-laying females were put into each of two dishes in the evening. In one dish was placed ordinary water, in the other water that had been oxygenated in the manner described in the preceding experiments. The females were removed after about an hour, but the eggs which they had laid were left in the dishes. The oxygenated water in one dish was then removed and replaced with fresh oxygenated water, and the dish was set under a bell jar in an atmosphere containing an excess of oxygen (40 or 60 per cent.). To insure that mechanical disturbance or

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accumulation of metabolic products did not affect the time of development of the eggs unequally, the water in the control dish was also drawn off after the rotifers were removed and replaced with fresh water. This dish was then set under a bell jar in air.

The next morning the two dishes were examined at intervals of 20 to 30 minutes, and the young rotifers removed and counted as they hatched. In this way the approximate time of development of the eggs was determined. In Table III. these times are collected in groups, to the nearest half hour. When these figures are treated statistically, they compare with one another as in Table IV.

TABLE III.

Showing the Time Required for Development of Eggs in Oxygenated Water and in Untreated Water.

	Number of Eggs Hatching in (Hours).						
	11.5.	12 0.	12.5.	13.0.	13.5.	14.0.	14.5.
Oxygenated water	7	10	15	23	9	1	
Untreated water	7	8	12	15	14	4	1

TABLE IV.

Showing the Time of Development of Eggs in Oxygenated Water and in Untreated Water. Statistical Treatment of Data in Table III.

	In Oxygenated Water.	In Untreated Water.	Difference.
Mean time of development in hours	12.654 ± 0.104	12.803 ± 0.129	0.149 ± 0.165
Standard deviation of time of development	1.239 ± 0.074	1.497 ± 0.091	0.258 ± 0.117

The eggs in oxygenated water hatched in a trifle shorter time, and somewhat more uniformly, though the difference in each case is so small that it may be insignificant. The greater uniformity of the time of development in oxygenated water (second line of Table IV.) is not improbably significant.

Effect of Oxygen upon the Contraction of the Foot Muscles.

Rotifers were placed in water saturated with an atmosphere containing 40 per cent. of oxygen, under a bell jar containing a similar atmosphere, and kept there 24 hours. They were then

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removed and promptly killed in Bouin's fluid. Other rotifers from the same families were kept in ordinary water, under a bell jar in air, for 24 hours, then killed in Bouin's fluid.

The contraction of the foot muscles was noted in accordance with the following arbitrarily chosen degrees of contraction: (0) foot fully extended; (1) foot slightly contracted, toes bent to one side, but still visible; (2) foot considerably contracted, toes wholly concealed, but contraction limited to small region near toes; and (3) foot greatly contracted, region of contraction much greater than in preceding class. It is to be noted that this classification is not the same as that proposed for the English and Nebraska rotifers in my former paper (Shull, 1915). The descriptions there given were not applicable to the rotifers used in these experiments.

The degrees of contraction of the foot muscles of the two lots of rotifers is tabulated in Table V., and the statistical comparison of the figures in Table VI.

TABLE V.

Showing the Degree of Contraction of the Foot Muscles of Rotifers Kept in Oxygenated Water, and in Untreated Water, and Then Killed in Bouin's Fluid.

Degree of Foot Contraction.	Number of Rotifers of Given Foot Contraction.	
	In Oxygenated Water.	In Untreated Water.
0	35	21
1	97	111
2	64	64
3	2	2

TABLE VI.

Statistical Comparison of Data in Table V.

	In Oxygenated Water.	In Untreated Water.	Difference.
Mean foot contraction.....	1.16 ± 0.034	1.27 ± 0.030	0.11 ± 0.046
Standard deviation of foot contraction.....	0.64 ± 0.021	0.71 ± 0.024	0.07 ± 0.031

The statistical treatment is based on the assumption that each degree of contraction of the foot muscles differs from the degrees next to it by unity.

As shown in Table VI. there is less contraction of the foot

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muscles in oxygenated water than in untreated water, but the difference is rather small compared with its probable error and in view of the crudity of the method of measurement may be without significance.

Effect of Temperature upon the Laying of Eggs at Surface and Bottom.

Equal numbers of rotifers were placed in a number of dishes of water. Some of the dishes were kept at room temperature which was fairly constant at 20° to 22° C. Other dishes were kept much cooler by setting them on a window sill outside. A thermometer was kept beside the latter dishes, and was fre-

TABLE VII.

The Number of Eggs Laid at the Surface Film and at the Bottom at Room Temperature and at Considerably Lower Temperatures.

Date.	Room Temperature.		Low Temperature.		
	Number of Eggs at Surface.	Number of Eggs at Bottom.	Number of Eggs at Surface.	Number of Eggs at Bottom.	Temperature in Degrees C.
Nov. 28.....	44	74	1	6	8
Nov. 29.....	20	59	0	14	7
			0	8	
			0	9	
			0	9	
Dec. 3.....	8	20	0	6	4
			0	10	
			0	4	
			0	2	
			0	0	
Dec. 4.....	15	22	0	0	2
			0	1	
			0	1	
			0	0	
Dec. 7.....	74	20	0	3	3
			0	1	
			0	1	
Dec. 8.....	6	20	3	7	12
			2	19	
			0	0	4
			0	3	
Totals.....	167	215	6	104	
Percentage at surface....	43.7		5.5		

quently read. In Table VII., which gives the number of eggs laid at the surface film and at the bottom, the stated temperature

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of these cool dishes is an estimated average. Because fewer eggs were to be expected at such low temperatures, several dishes were kept at low temperature for every one at room temperature.

There is no question here that the eggs are laid more largely at the surface when the temperature is high. The experiment was repeated on a small scale with the same result.

Effect of Temperature upon the Viability of the Fertilized Eggs.

Freezing.—Freezing of fertilized eggs was designed as a practical measure only, in order to induce those eggs to hatch which would not otherwise hatch. The attempt failed, however, as shown by the following tests.

In the first experiment about 244 eggs that remained unhatched after hatching had ceased for 12 days in the lot of eggs to which they belonged, were divided into two approximately equal groups. One group was frozen over night by immersing, in a closed vessel, in a brine-ice mixture and the other was kept in water at ordinary temperature. Of the frozen lot none hatched in 19 days thereafter. Of the control lot at room temperature, one hatched in 10 days after the beginning of the experiment, none thereafter. Freezing did not facilitate the hatching.

In the second experiment about 230 eggs that remained unhatched for 14 days after hatching of the eggs in the same lot had practically ceased were similarly divided into two groups, one of which was frozen over night and the other kept at room temperature. None of these eggs in either lot hatched in 19 days, after which time observation ceased.

From these experiments it appears that fertilized eggs not ordinarily capable of hatching can not be made to hatch by freezing.

Low Temperature Above Freezing.—Two lots of fertilized eggs from the same source and of approximately equal numbers were kept at different temperatures from the time they were laid until hatching was nearly complete. The eggs were laid between November 29 and December 8. One lot was kept at room temperature. The other was set outside on a window sill where daytime temperature, as shown by a thermometer placed beside

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the dishes, ranged from 2° to 12° C. The air temperature was much below this, but was moderated in the location of the eggs by a steam radiator near the window sill inside. It appears that the water in which the eggs were placed never froze and hatching occurred during this period of low temperature. From December 22 to December 25 the atmospheric temperature was considerably above freezing but was much colder thereafter. On January 3 the "cold" dish was removed to room temperature, but hatching had been nearly completed before that time. Table VIII. shows the number of eggs that hatched.

TABLE VIII.

Showing the Effect of Room Temperature and Lower Temperatures Upon the Hatching of the Fertilized Eggs of Hydatina Senta.

Date of Beginning Experiment.	Warm.			Cold.		
	Number of Eggs.	Date of Hatching.	Number Hatched.	Number of Eggs.	Date of Hatching.	Number Hatched.
Nov. 29....	44	Dec. 6	1	43	Dec. 23	1
Dec. 2....	10	Dec. 7	1	10	Dec. 23	2
					Dec. 24	2
					Jan. 5	1
Dec. 8....	43	Dec. 10	2	44	Dec. 10	1
					Dec. 12	2
					Dec. 24	1
Total number hatching.....			4			10

Low temperature appears to favor the hatching of the eggs, not as an after effect, but during the period of low temperature.

Effect of Oxygen upon the Viability of the Fertilized Eggs.

Each of four lots of eggs from a single source was divided into two equal parts. One was placed in water of high oxygen content (saturated with an atmosphere of which either 40 per cent. or 60 per cent. was oxygen) and set under a bell jar enclosing an atmosphere containing the same proportion of oxygen as that with which the water was originally saturated. Atmospheres of 40 per cent. oxygen were used until July 7, 60 per cent. thereafter. This dish was removed from the bell jar daily and examined for hatching rotifers. The water was drawn off the eggs after examination and replaced with fresh oxygenated water and the dish returned to the bell jar.

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The other dish was filled with untreated water which was drawn off daily and replaced with fresh water.

The hatching of the eggs is recorded in Table IX.

TABLE IX.

Showing the Effect of Oxygenation of the Water Upon the Hatching of the Fertilized Eggs of Hydatina Senta.

Experiment.	Date of Starting Experiment.	Oxygenated Water.			Untreated Water.		
		Number of Eggs.	Date.	Number Hatching.	Number of Eggs.	Date.	Number Hatching.
A.....	July 1, 1915	24	July 3	1	24	July 2	1
			7	1		7	1
			9	1		8	2
						12	1
				3			5
B.....	July 2, 1915	30	July 6	4	30	July 5	4
			10	1		8	1
			21	1		9	1
						10	1
						17	1
				6			8
C.....	July 4, 1915	79	July 5	1	78	July 8	1
			9	4		9	5
			10	5		10	4
			11	2		11	1
			12	3		12	3
						13	1
						14	1
				15			16
D.....	July 7, 1915	100	July 10	1	100	July 8	1
			11	2		9	1
			12	2		10	1
			12	5		11	3
			14	2		12	1
						13	8
						14	3
						21	1
						23	1
				12			20
Grand total.....	.....			36	.....		49

The eggs in oxygenated water show a somewhat lower viability in every case.

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Effect of Drying upon the Viability of the Fertilized Eggs.

Dried for a Short Period.—A lot of cross-fertilized eggs—eggs laid by females of line B fertilized by males of line A of my former experiments (Shull, 1913)—were kept for seven weeks to allow hatching to take place. Practically all the hatching occurred in the second to fourth weeks of this period, almost none in the last three weeks. The 446 eggs (about two thirds of the original lot) which remained unhatched after seven weeks were divided into two nearly equal groups. One half was allowed to become dry December 8 and remain dry about 13 hours, after which it was remoistened. The other half was kept wet. The subsequent hatching of eggs from these two groups is recorded in Table X.

TABLE X.

Showing the Effect of Drying Eggs for a Short Period Upon the Proportion that Hatch in a Cross-Fertilized Lot of Eggs of Hydatina Senta.

Eggs Kept Wet.		Eggs Dried and Remoistened.	
Date.	Number Hatching.	Date.	Number Hatching.
Dec. 9	0		
10	0	Dec. 10	0
11	1	11	0
12	0	12	0
13	0	13	2
14	0	14	3
15	0	15	1
16	0	16	8
17	0	17	4
18	1	18	5
19	0	19	4
20	0	20	7
Total.....	2		34

Observations necessarily stopped December 20, but it seems likely that even more would have hatched among the eggs that were dried. Drying for a short period either favors hatching of eggs that would not otherwise have hatched or hastens the hatching of eggs whose hatching would otherwise have been spread over a long period.

The above experiment was repeated with a lot of eggs from the reciprocal cross of the foregoing—eggs laid by females of line A fertilized by males of line B. The original lot of eggs was kept seven weeks to allow of hatching. During the last three weeks

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of that time very little hatching occurred. The 238 eggs that remained (less than half of the original lot) were divided into two equal parts, one of which was allowed to become dry and remain so for 13 hours on December 4. The subsequent hatching is recorded in Table XI. The results confirm the conclusions drawn from Table X.

TABLE XI.

Showing the Effect of Drying Eggs for a Short Period Upon the Proportion that Hatch in a Cross-Fertilized Lot of Eggs of Hydatina Senta. The Cross was the Reciprocal of that in Table X.

Eggs Kept Wet.		Eggs Dried and Remoistened.	
Date.	Number Hatching.	Date.	Number Hatching.
Dec. 5–20	0	Dec. 7–15	0
		16	1
		17	1
		18	16
		19	2
		20	1
Total.....	0		21

In another experiment of this kind inbred eggs (eggs laid by females fertilized by males of the same line) of a line in which a relatively small proportion of the eggs normally hatched were used. The eggs were kept five weeks, during the last 12 days of which time no eggs hatched. The eggs that remained unhatched were divided into two lots which, by mistake, were made unequal. One lot, consisting of about 130 eggs, was dried over night; the other of 106 eggs, was kept wet. The subsequent hatching of these eggs is recorded in Table XII.

TABLE XII.

Showing the Effect of Drying for a Short Period Upon the Hatching of Inbred Eggs of Hydatina Senta.

Eggs Kept Wet.		Eggs Dried and Remoistened.	
Date.	Number Hatching.	Date.	Number Hatching.
Feb. 25 to Mar. 8 ..	0	Feb. 24 to Mar. 4 .	0
Mar. 9.....	1	Mar. 5.....	1
Mar. 10 to 14.....	0	Mar. 6 to 14.....	0

Unlike the cross-fertilized eggs of Tables X. and XI., drying for a few hours neither increased the number of eggs that hatched nor hastened their time of hatching.

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The foregoing experiment was repeated with inbred eggs of a line that regularly hatched more than half its eggs. The eggs remaining unhatched after five weeks were divided into two lots, one of which was dried over night and then remoistened. Though both were kept three weeks, not one egg in either lot hatched. Drying neither increased nor hastened the hatching.

Dried for Periods of Moderate Length.—These experiments differed from the foregoing in that all of the eggs were dried in one half of the experiment, instead of only those which failed to hatch under ordinary conditions. Inbred eggs were used, and drying occurred about the time when hatching was due to begin, that is, a week after the eggs were laid. Hatching began three or four days after the eggs were remoistened. Observations were continued for a month after remoistening. One lot of eggs was kept wet as a control, one was dried over night, one dried two weeks, and a fourth dried four weeks. The experiment was performed three times. The totals, without daily records, are given in Table XIII. The second division of this table really belongs to the preceding section of this paper, since it involves only a short period of drying, but it seems best to retain it here for the sake of comparison.

TABLE XIII.

Showing the Effect of Drying for Various Periods Upon the Viability of the Fertilized Eggs of Hydatina Senta. Inbred Eggs Were Used.

Experiment.	Eggs Kept Wet.		Dried Over Night.		Dried Two Weeks.		Dried Four Weeks.	
	Number of Eggs.	Number Hatching.	Number of Eggs.	Number Hatching.	Number of Eggs.	Number Hatching.	Number of Eggs.	Number Hatching.
A	75	40	78	35	76	18	72	0
B	55	26	41	6	50	12	53	0
C	39	23	33	19	38	6	27	0
Total.....	169	89	152	60	164	36	152	0
Percentage hatching..	52.6		39.4		21.9		0.0	

There is plain indication in these results that drying, even for a short time, reduces the number of eggs that will hatch when again placed in water; and that the longer the period of desiccation, up to the limit of complete inhibition, the fewer the eggs that hatch.

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Dried for a Long Period.—No experiment with control, involving a longer period of desiccation than four weeks, was performed; but that some eggs could withstand longer desiccation was shown. One lot of 344 cross-fertilized eggs and another of 100 inbred eggs, all from sources not used in the experiments described in this paper, were kept in dried condition from June 29 to March 23, or about nine months, when they were remoistened. In about three weeks thereafter three eggs of the first group and one of the second hatched.

All these eggs simply rested on the bottom of a watch glass when dried. It is not improbable that when the eggs, on drying, are supported by mud or sand they may remain desiccated longer and still hatch when remoistened. But even in mud the possible period of desiccation is not indefinitely long; for out of a lot of fertilized eggs in dried mud I have secured numerous young rotifers after three months of desiccation, but no eggs hatched from this lot after two years.

Summary and Discussion.

In former papers (Shull, 1913, 1915) the inheritance of size of parthenogenetic eggs, the time of development of parthenogenetic eggs, the place of laying parthenogenetic eggs (surface film or bottom), the viability of fertilized eggs (proportion that hatch), and the contractility of foot muscles was described. In this paper is shown to what extent these inherited characters may have been modified by such external agencies as temperature, oxygen, and desiccation.

It was found that if the water in which the rotifers live was exposed to an atmosphere containing more than the usual proportion of oxygen, a greater proportion of the eggs were laid at the bottom of the vessel. Under ordinary conditions the rotifers probably come to the surface because of the greater quantity of dissolved oxygen there. Those lines which normally lay their eggs mostly at the bottom probably either require less oxygen or get their oxygen more easily than lines which lay their eggs at the surface film. The hereditary character involved may therefore be the oxygen requirement or the permeability to oxygen.

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If the rotifers were kept at a low temperature, their eggs were laid more largely at the bottom. This may be due to the greater concentration of oxygen in the water at low temperature, so that it is unnecessary to come to the surface so much; or to the low metabolism and hence low oxygen requirement of the animals; or to both of these reasons.

Excess of oxygen increased the size of the parthenogenetic eggs only very slightly, or not at all. Excess of oxygen decreased the time of egg development only very slightly, or not at all. It may have made the time of development a trifle more uniform, though this is hardly proven.

Rotifers kept in oxygenated water showed, when killed in Bouin's fluid, a trifle less contraction of the foot muscles than did other rotifers, though the difference was too small to constitute a proof of the action of oxygen.

Since the place of laying the eggs (surface or bottom) was so much more greatly affected by oxygen than were the other three characters tested, it would seem rather improbable even if the genetic results (Shull, 1915) had permitted such an assumption, that the several characters in which the English and Nebraska lines differ as described in the introduction to this paper, were really but a single character with several manifestations. The results described in this paper, therefore, harmonize with the genetic results previously obtained.

Freezing fertilized eggs that had remained unhatched for five to seven weeks did not induce any of them to hatch later. However, when eggs were kept from the time of laying at a low and variable temperature above freezing, more of them hatched than when kept at room temperature.

When fertilized eggs were kept in oxygenated water, a somewhat smaller proportion of them hatched than in untreated water.

Cross-fertilized eggs which had remained for seven weeks under ordinary conditions without hatching were dried over night and then remoistened. A considerable number of them were thereby caused to hatch. Inbred eggs, however, when tested in the same way, did not respond to drying. Even inbred eggs that were dried shortly after they were laid (not merely

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those which failed to hatch otherwise) did not show any increase in the proportion of viable eggs. Indeed, drying had just the opposite effect on inbred eggs. Even when the eggs were kept dry only a few hours, the percentage of them that hatched was reduced; and the longer the eggs were kept dried the fewer of them hatched. Those that remained dry for four weeks did not hatch at all.

Other lines were not as sensitive to drying, for out of one lot of eggs that were dry for nine months, several eggs hatched when remoistened.

In view of the results of desiccation of inbred eggs, it is conceivable that the hatching of cross-fertilized eggs after drying was due merely to the hastening of the development of eggs whose hatching would otherwise have been spread over a long period. If we had for comparison only the experiments with inbred eggs which were dried immediately after laying, and those with cross-fertilized eggs that were dried after they had been allowed abundant time in which to hatch and had not done so, the conclusion just stated would seem not merely conceivable, but probable. However, since inbred eggs were also dried after their normal hatching period was past, and failed to hatch subsequently, whereas cross-fertilized eggs thus treated did hatch, I am inclined to believe that drying for a few hours actually caused some cross-fertilized eggs to hatch which would not have done so without drying. From the physiological viewpoint, such a difference between inbred eggs and cross-fertilized eggs is not at all improbable.

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THE EFFECT OF SELECTION UPON THE SEX-RATIO IN DROSOPHILA AMPELOPHILA.

DON C. WARREN.

FROM THE ZOÖLOGICAL LABORATORY OF INDIANA UNIVERSITY.[1]

It is a well-known fact that sex-ratios in most animals approximate equality. The ratio is seldom one of exact equality, however, and the preponderance may be in favor either of the males or the females. The variation from equality is fairly constant for the species. For man the ratio has been found to be 100 females to 105 males; for the horse, 100 to 98; for the cow, 100 to 107; for the sheep, 100 to 97; for the pig, 100 to 111; for the dove, 100 to 105; and for the hen, 100 to 94. These variations are so constant that they cannot be attributed to chance and they are irregularities for which our present theories of sex determination offer no adequate explanation.

The writer has attempted to determine the sex-ratio of the fruit fly, *Drosophila ampelophila*. The determination was made from three unrelated stocks in which the best possible environmental conditions were provided. All matings were in single pairs. Over 35,000 flies were examined and the ratio was found to be 100 females to 95 males.

Moenkhaus tested the effect of selection upon the sex-ratio of *Drosophila ampelophila* and decided that the sex-ratio in this species is "amenable to selection." If it be true that the relative number of males and females in a strain can be varied by selection, the present theories of sex determination must be somewhat modified. Although this fact would not necessarily disprove the theories of sex determination, it would necessitate the assumption of an hypothesis of selective fertilization or of differential mortality or viability of the determining elements.

In view of the vital bearing of Moenkhaus's findings upon the theories of sex determination, it has been deemed worth while to repeat his work.

[1] Contribution No. 159.

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Methods and Material.

The selection experiments were carried out upon three unrelated stocks. The stock for the first experiment was collected in Bloomington, Ind.; for the second, in Saratoga, Ind.; and for the third, in Warsaw, Ind. In the first two cases the experiments were started as soon as the stocks were collected from nature but the Warsaw stock was inbred in the laboratory for about six months before the experiment was started. At all times the greatest care was exercised to prevent contamination. The flies were provided with an abundance of food and care was taken to examine all the offspring of each pair. All matings were single pair matings.

Each experiment was conducted upon the offspring of a single pair of flies, that is, for the first experiment, a virgin female was selected from the Bloomington stock and mated to a brother. From among the offspring of this pair of flies, the parents of the two strains were obtained. The one strain in which there was selection for a relatively high number of females in comparison to the number of males, has been called the "high" strain and the one in which the selection was in the opposite direction, has been called the "low" strain. Before selection was started, a sufficient number of flies were examined to obtain a fair estimate of the sex-ratio of the stock under consideration. Moenkhaus's high and low strains were not offspring of a single pair of flies but were obtained by selecting from nature, pairs which had unusual ratios in the desired direction. Furthermore, he knew nothing of the original sex-ratio of his strains before selection began.

In each generation an attempt was made to examine the offspring of ten pairs in each strain. Unsuccessful matings, usually due to poor food conditions, sometimes prevented this. In each generation, the pair which gave the most extreme ratio in the desired direction was used as the parent of the succeeding generation. At times, technical difficulties prevented the most extreme pair being used and in these cases the next best was used. Pairs showing extreme ratios but producing a small number of offspring were not used. Since it was not possible to know which pair had the most desirable ratio till all of the offspring of all the pairs in a generation were examined, the technique of the

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experiment was made somewhat difficult. This might have been overcome by isolating some males and females from each mating and then, after the counts for that generation were completed, mating the offspring of the pair giving the most extreme ratio. This would have prolonged the experiment, so to avoid delay, at about the eighth or ninth day after the young had begun to emerge, fifteen pairs were mated from among the offspring of each mating which was being examined. In this way, by the time one generation was finished, the next was almost ready to begin to emerge. Since ten matings in each strain were examined, this necessitated the mating of about300 pairs of flies in each generation. A control was also carried in each experiment in which a few hundred flies were examined each generation.

Preliminary Experiment.

This experiment was to test whether the age of the parents would in any way influence the relative number of males and females produced. Several matings were made and the adults

Table I.

Showing Results of Experiment to Test the Effect of Age of Parent Upon the Sex-Ratio.

Culture Number.	Original Mating 1 Day Old.		First Transfer 11 Days Old.		Second Transfer 18 Days Old.		Third Transfer 26 Days Old.		Fourth Transfer 34 Days Old.		Fifth Transfer 42 Days Old.		Sixth Transfer 50 Days Old.		Seventh Transfer 58 Days Old.		Eighth Transfer 62 Days Old.		Totals.	
	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.
202A2.	56	68	112	110	105	111	51	40											324	329
203A2.	93	92	110	128	60	89	126	124	172	152	49	60							610	645
203A2.	156	162	135	150	58	60	78	89	136	123	91	93							654	677
204A1.	232	194	140	146	20	24													392	364
204A2.	223	234	155	156	157	151	115	115											650	656
204A2.	134	128	46	70	38	37													218	235
205A2.	214	202	109	114	86	104	115	110	12	12									536	542
205A2.	187	161	140	130	103	113	114	107											544	511
206A1.	68	58	80	55	75	77	78	76	94	89	72	95	126	118	45	45	10	5	648	618
206A2.	149	152	173	176	79	126	135	106	152	130	34	23							722	713
206A2.	173	164	179	207	141	134	129	162	156	159	183	203	71	91					1032	1120
207A1.	90	87	153	143	81	90	100	100											424	420
207A2.	120	118	129	123	86	83	84	69											419	393

were transferred to new bottles every eight days as long as they both lived. The age of the parents does not influence the sex-ratio as is shown by the results given in Table I.

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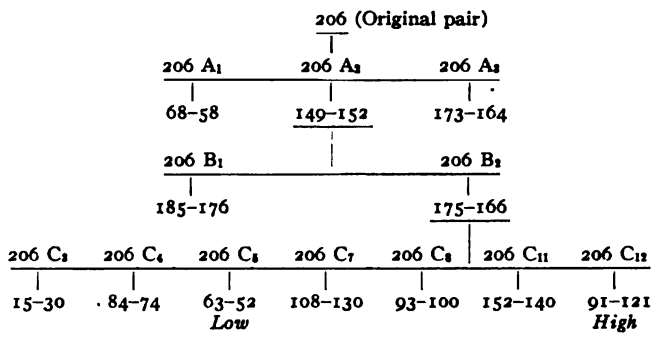
Selection Experiment I. (Bloomington Stock.)

From a stock of flies collected in Bloomington, a virgin female was selected and mated to a male from the same stock. From the offspring of this pair of flies the high and low strains were started. Before selection was begun, 2,936 flies were examined for sex. The ratio was 1,456 males to 1,480 females, or 1 male to 1.06 females. The ratio here is stated in just the reverse order from the customary form but since Moenkhaus used the reverse order, the ratios in the present discussion will be stated likewise to make the two experiments more comparable. Table II. gives the history of the origin of the high and low strains.

Table II.[1]

Showing Origin of the "High" and "Low" Strains of the Bloomington Stock.



Tables III. and IV. give the history of the high and low strains. There is no indication of the ratios having changed in the direction of the selection. In fact, in the high strain where there was selection for a relatively high number of females, the totals show a relatively lower number of females than in the low strain. The totals for the high strain were 8,837 males to 8,942 females or a ratio of 1 male to 1.012 females while in the low strain there were 8,368 males to 9,091 females or a ratio of 1 male to 1.086 females.

[1] In all tables where two numbers are expressed with a dash between them, as 68–58, the number to the left of the dash always represents the male count and the number to the right, the female count.

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TABLE III.

Showing Summary of the "High" Strain of the Bloomington Stock. Experiment I.

91 ♂–121 ♀ (1 : 1.33)
(From Mating 206C_{12})

Generation.											Totals.
A											
	2	4	5	6	7	8	9	10	11	13	
B	145–150	200–224	216–229	188–198	179–177	229–183	154–162	99–85	111–116	121–140	1642–1664
	1 : 1.03	1 : 1.12	1 : 1.06	1 : 1.05	1 : .99	1 : .80	1 : 1.05	1 : .85	1 : 1.05	1 : 1.16	1 : 1.013
	1	3	4	5	6	8	10	12		13	
C	78–65	106–118	153–148	167–179	53–53	176–165	143–136	127–115		42–69	1045–1048
	1 : .83	1 : 1.11	1 : .97	1 : 1.07	1 : 1	1 : .94	1 : .95	1 : .91		1 : 1.64	1 : 1.003
	2	3	5	6	7	9	10	11	12	14	
D	201–233	83–83	115–110	92–90	221–204	117–125	95–104	37–35	139–160	284–254	1384–1399
	1 : 1.16	1 : 1.0	1 : .96	1 : .99	1 : .92	1 : 1.07	1 : 1.09	1 : .95	1 : 1.15	1 : .89	1 : 1.01
	1	2	3	4	5	6	7	10	12	13	
E	117–99	184–176	223–225	194–173	302–279	179–180	252–278	93–111	195–194	36–31	1775–1746
	1 : .85	1 : .96	1 : 1.01	1 : .89	1 : .92	1 : 1.01	1 : 1.1	1 : 1.19	1 : .99	1 : .86	1 : .984
	1	2	3	4	5	7	9	12	13	14	
F	179–177	208–188	79–70	98–128	188–154	124–142	149–159	158–154	113–139	52–61	1348–1372
	1 : .988	1 : .90	1 : .89	1 : 1.31	1 : .82	1 : 1.15	1 : 1.07	1 : .97	1 : 1.241	1 : 1.18	1 : 1.018
	1	2	5	6	8	10	11	12	14	15	
G	85–96	166–168	31–55	164–174	219–223	125–135	144–143	70–80	111–119	86–96	1201–1289
	1 : 1.13	1 : 1.01	1 : 1.77	1 : 1.06	1 : 1.02	1 : 1.08	1 : .99	1 : 1.143	1 : 1.07	1 : 1.12	1 : 1.073
	3			4		8	9	10			
H	95–101			116–103		64–57	50–35	117–128			442–424
	1 : 1.06			1 : .89		1 : .89	1 : .70	1 : 1.09			1 : .959

Grand Total.....8837–8942
1 : 1.012

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TABLE IV.

Showing Summary of the "Low" Strain of the Bloomington Stock. Experiment I.

Generation.											
A	63 ♂–52 ♀ (1 : .83) (From Mating 206C₈)										
	1	3	4	5	7	8	9	10			Totals.
B	164–228	227–248	109–113	138–146	190–187	35–88	185–184	191–197			1239–1391
	1 : 1.39	1 : 1.09	1 : 1.04	1 : 1.06	1 : .98	1 : 2.51	1 : .99	1 : 1.03			1 : 1.123
	1	4	5	7	8	9	10	11			
C	123–135	51–53	291–327	105–119	120–114	60–44	176–181	52–71			978–1044
	1 : 1.1	1 : 1.04	1 : 1.12	1 : 1.13	1 : .95	1 : .73	1 : 1.03	1 : 1.37			1 : 1.067
	2	6	7	9	10	11	12	13	14	15	
D	175–207	153–128	136–160	130–126	133–158	192–196	85–104	225–261	135–158	181–199	1545–1697
	1 : 1.18	1 : .84	1 : 1.18	1 : .97	1 : 1.19	1 : 1.03	1 : 1.22	1 : 1.16	1 : 1.17	1 : 1.2	1 : 1.098
	1	2	4	5	8	9	10	11	12	15	
E	176–141	125–123	138–131	141–135	151–151	139–142	285–277	167–141	193–234	180–180	1695–1655
	1 : .8	1 : .98	1 : .95	1 : .96	1 : 1	1 : 1.02	1 : .97	1 : .84	1 : 1.21	1 : 1	1 : .976
	1	2	3	4	6	7	9	11	12	14	
F	275–252	174–167	145–179	115–105	173–190	194–180	164–264	246–266	219–215	181–202	1886–2020
	1 : .92	1 : .96	1 : 1.23	1 : .91	1 : 1.10	1 : .93	1 : 1.61	1 : 1.08	1 : .981	1 : 1.11	1 : 1.071
	1	6	9	11	14	15					
G	114–134	84–180	89–83	205–190	37–30	84–132					613–749
	1 : 1.18	1 : 2.14	1 : .93	1 : .93	1 : .81	1 : 1.57					1 : 1.221
	1	4	5	7	8	10	12	13	14	17	
H	52–64	56–72	59–87	48–70	38–47	47–53	8–14	9–9	45–61	50–58	412–535
	1 : 1.23	1 : 1.29	1 : 1.47	1 : 1.46	1 : 1.24	1 : 1.13	1 : 1.75	1 : 1	1 : 1.36	1 : 1.16	1 : 1.298

Grand Total.....8368–9091
1 : 1.086

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SELECTION EXPERIMENT II. (Saratoga Stock.)

In the first experiment, a much larger number of flies were examined than were examined by Moenkhaus. But since opposite results were obtained, a criticism might be offered that the writer, by chance, selected a stock which was not affected by selection. So the second experiment was made upon a new and unrelated stock. This stock was collected at Saratoga, Indiana. Fifty matings were made from among the offspring of a single pair. The extremes of these fifty were used as parents of the high and low strains. The counts of these fifty pairs are given in Table V. There were 21,019 flies examined before selection began and the stock showed a ratio of 1:1.0512, or 10, 247 males to 10,772 females.

TABLE V.

Showing the Origin of the "High" and "Low" Strains of the Saratoga Stock.

♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.
264	235[1]	202	222	252	241	213	248	179	149
157	165	246	236	157	190	187	168	266	263
208	230	290	280	163	202	241	235	224	234
285	256	192	232	214	237	232	257	109	129
137	160	218	223	176	188	245	205	184	171
173	190	187	217	243	282	75	106	226	206
193	187	251	222	160	189	245	225	195	233
155	168	192	207	200	280	188	171	183	168
252	236	181	307[2]	212	275	129	175	226	222
288	271	183	187	200	225	235	247	234	220
Grand Total..... 10,247 ♂'s to 10,772 ♀'s									

The results of the selection in this experiment are given in Tables VI. and VII. In this stock there were greater individual variations from the normal sex-ratios in each direction but they did not breed true. The totals show the high strain to have a higher relative number of females than the low strain. They are as follows: for the high strain, 7,377 males to 8,365 females, or a ratio of 1:1.134; for the low strain, 10,923 males to 11,246 females or a ratio of 1:1.029. Considering these totals, it might seem that something had been accomplished by selection but when the totals are considered generation by generation the results do not seem so conclusive. If selection has been the factor which

[1] From this mating originated the "low" strain.

[2] From this mating originated the "high" strain.

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TABLE VI.

Showing Summary of the "Low" Strain of the Saratoga Stock. Experiment II.

Generation.											
A	264 ♂–235 ♀ (1 : .89)										
	1	2	3	4	5	6	7	8	9	10	Totals.
B	146–150	159–202	199–152	199–213	131–129	83–91	81–78	109–119	108–100	107–121	1322–1355
	1 : 1.03	1 : 1.27	1 : .76	1 : 1.07	1 : .99	1 : 1.10	1 : .96	1 : 1.09	1 : .926	1 : 1.13	1 : 1.024
	1	2	3	4	5	6	7		8	10	
C	144–161	135–143	210–208	132–130	138–149	157–138	224–251		45–44	90–84	1275–1308
	1 : 1.12	1 : 1.06	1 : .99	1 : .98	1 : 1.08	1 : .88	1 : 1.12		1 : .98	1 : .93	1 : 1.0259
	1	2	3	4	5	6	7	8	9	10	
D	270–294	212–242	174–135	193–217	223–211	256–246	67–81	219–215	308–308	267–263	2189–2212
	1 : 1.09	1 : 1.14	1 : .78	1 : 1.12	1 : .95	1 : .96	1 : 1.21	1 : .98	1 : 1	1 : .985	1 : 1.0105
	1	2	3	4	5	6	7	8	9	10	
E	137–136	106–115	50–65	146–169	115–130	153–153	109–138	206–188	32–39	160–149	1214–1282
	1 : .99	1 : 1.08	1 : 1.3	1 : 1.16	1 : 1.13	1 : 1	1 : 1.27	1 : .91	1 : 1.22	1 : .931	1 : 1.056
	2	3	4	5	6						
F	166–176	199–213	72–84	171–210	62–37						670–720
	1 : 1.06	1 : 1.07	1 : 1.17	1 : 1.23	1 : .60						1 : 1.0746
	1	2	3	4	5	6	7	8	9	10	
G	43–41	101–97	130–137	124–121	79–90	154–170	90–103	148–156	68–67	205–213	1142–1195
	1 : .95	1 : .96	1 : 1.05	1 : .98	1 : 1.14	1 : 1.10	1 : 1.14	1 : 1.05	1 : .985	1 : 1.039	1 : 1.0464
	1	2	3	4	5	6	7	8	9	10	
H	199–197	172–169	144–152	169–179	151–152	124–141	92–101	210–225	163–185	114–107	1538–1608
	1 : .99	1 : .98	1 : 1.06	1 : 1.06	1 : 1.01	1 : 1.14	1 : 1.10	1 : 1.07	1 : 1.135	1 : .938	1 : 1.0455
	1	2	3	4	5	6	7	8	9	10	
I	184–178	182–183	164–137	184–180	74–97	111–110	187–216	136–116	177–168	174–181	1573–1566
	1 : .967	1 : 1.005	1 : .835	1 : .978	1 : 1.31	1 : .991	1 : 1.155	1 : .853	1 : .949	1 : 1.04	1 : .996

Grand Total.....10,923–11,246
1 : 1.020

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TABLE VII.

Showing Summary of the "High" Strain of the Saratoga Stock. Experiment II.

Generation.											Totals.
A	181 ♂–307 ♀ (*1 : 1.696*)										
	1	2	3	4	5	6	7	8	9	10	
B	149–163	145–152	147–212	208–194	201–178	172–198	143–149	240–240	22–22	207–231	1634–1739
	1 : 1.09	*1 : 1.05*	*1 : 1.442*	*1 : .932*	*1 : .885*	*1 : 1.151*	*1 : 1.04*	*1 : 1*	*1 : 1*	*1 : 1.116*	*1 : 1.064*
	2		4		6	8	9	10			
C	72–81		122–109		163–158	117–118	106–148	144–136			724–750
	1 : 1.125		*1 : .893*		*1 : .969*	*1 : 1.008*	*1 : 1.396*	*1 : .944*			*1 : 1.036*
	1	2	3	4	5	6	7	8	9	10	
D	171–192	132–157	119–203	189–324	191–221	175–212	140–222	127–189	97–169	136–149	1477–2038
	1 : 1.123	*1 : 1.189*	*1 : 1.706*	*1 : 1.714*	*1 : 1.157*	*1 : 1.211*	*1 : 1.586*	*1 : 1.488*	*1 : 1.742*	*1 : 1.096*	*1 : 1.3798*
	1	2	3	4	5	6	7	8	9	10	
E	107–143	111–180	152–144	93–109	88–104	207–181	70–106	168–228	98–99	33–43	1127–1337
	1 : 1.34	*1 : 1.62*	*1 : .95*	*1 : 1.17*	*1 : 1.18*	*1 : .874*	*1 : 1.51*	*1 : 1.36*	*1 : 1.01*	*1 : 1.30*	*1 : 1.186*
	2	3	4	5	6	7	8	9		10	
F	258–276	50–37	118–107	119–102	260–221	81–104	33–28	163–206		49–56	1131–1137
	1 : 1.07	*1 : .74*	*1 : .907*	*1 : .86*	*1 : .85*	*1 : 1.284*	*1 : .85*	*1 : 1.264*		*1 : 1.14*	*1 : 1.0053*
	1	2	3	4	5	7	8	9			
G	33–84	82–111	152–138	32–41	78–94	42–38	27–30	89–91			536–627
	1 : 2.55	*1 : 1.35*	*1 : .91*	*1 : 1.28*	*1 : 1.21*	*1 : .88*	*1 : 1.11*	*1 : 1.022*			*1 : 1.1698*
	2		3	4	5	6		9			
H	74–79		154–151	108–129	40–28	99–89		37–42			512–518
	1 : 1.068		*1 : .9805*	*1 : 1.19*	*1 : .70*	*1 : .899*		*1 : 1.135*			*1 : 1.0117*
	1		3		4	6		8			
I	63–81		22–15		60–56	68–49		23–18			236–219
	1 : 1.28		*1 : .68*		*1 : .93*	*1 : .72*		*1 : .78*			*1 : .928*

Grand Total.....7,377–8,365
1 : 1.134

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has made the difference between the two strains, the difference should be most evident in the later generations of the experiment. This is not true, for a moment's calculation will show that the last three generations in each strain do not show as extreme an average ratio in the desired direction as those which preceed tehm. Although the difference between the totals of the two strains may be sufficiently large to be considered significant, it seems more probable that it is a chance variation, especially since the first experiment showed a similar variation in the direction opposite selection.

Selection Experiment III. (Warsaw Stock.)

In order to make the work still more conclusive, selection was attempted upon a third stock. This stock was collected at Warsaw, Indiana, and kept as a stock culture in the laboratory for about six months before the experiment was started.

Here forty-two pairs were mated from among the offspring of a single pair and the extremes of these matings were used as the parents of the high and low strains.[3] There were 11,190 flies examined before the selection began, 5,448 males to 5,742 females, a ratio of 1 :.1.0539. The counts of these matings are given in Table VIII.

Table VIII.

Showing Origin of the "High" and "Low" Strains of the Warsaw Stock.

♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.	♂.	♀.
38	43	91	82	125	106	226	220	255	247
66	72	106	101	155	150	110	114	136	143
133	169	135	156	**235**	**238**[2]	80	72	84	77
192	208	41	39	102	105	74	93	94	100
85	75	105	128	48	61	114	129	187	200
147	**187**[1]	178	189	186	186	195	203	89	79
170	192	91	102	127	142	179	199	175	171
105	113	101	104	187	220	158	156		
125	119	106	114	112	138				

Grand Total..... 5,448 ♂'s to 5,742 ♀'s

[1] From this mating originated the "high" strain.

[2] From this mating originated the "low" strain.

[3] It will be seen in Table VIII. that the pair used as the parents of the low strain did not produce the most extreme ratio in that direction. This mistake was due to a mathematical error which was not checked up until it was too late to rectify the experiment.

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In the latter part of this experiment there was a diveation from the usual methods. The element of time was sacrificed to be able to make a larger number of matings in each generation. A number of males and females were isolated from each mating, being examined, and held to be mated after all of the counts for the generation were finished. By this method it was necessary to make matings only from the cultures which had given the most desirable ratios but in so doing a considerable amount of time was lost between each generation.

This third attempt to modify, by selection, the sex-ratio did not result in bringing about any very significant difference between the two strains. (Tables IX. and X. give the results of selection upon this stock.) The ratios computed from the totals in each strain showed a slight difference in the desired direction but this was so slight that it might be due to chance. Therefore it seems that no definite conclusions can be drawn from this experiment. In the low strain the totals were 9,673 males to 9,951 females, or a ratio of 1 : 1.0287 and in the high strain 12,327 males to 12,898 females, or a ratio of 1 : 1.0463.

Conclusion.

Since three distinct and unrelated stocks of flies show no significant effect of selection, it seems safe to conclude from these data that Moenkhaus's conclusion concerning the amenability of the sex-ratio in *Drosophila ampelophila* will not hold.

Discussion of Moenkhaus's Work.

Moenkhaus concludes from his work on the effect of selection on sex-ratios in *Drosophila ampelophila* that sex-ratios are "strongly transmissible and amenable to the process of selection." From his paper it is somewhat difficult to tell whether Moenkhaus intended to say that he had developed by selection, strains characterized by high and low female ratios or that by selection, he simply maintained a high and low strain which he found in nature. His results are given in the discussion which follows.

By way of explanation it might be said that Moenkhaus called the strain in which he selected for a relatively higher female ratio the "female" strain, and the one in which he selected in

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TABLE IX.

Showing Summary of the "Low" Strain of the Warsaw Stock. Experiment III.

235 ♂s–238 ♀s (1 : 1.013)

Generation.										Totals.
	2	3	4	5	6	7	9	10		
B	183–171	266–256	141–144	150–155	112–139	227–216	224–254	155–125		1458–1460
	1 : .934	1 : .962	1 : 1.02	1 : 1.033	1 : 1.24	1 : .951	1 : 1.13	1 : .806		1 : 1.0013
	1	2	3	4	5	6	7	8	9	10
C	222–207	234–252	231–242	105–112	211–254	164–193	247–246	158–160	96–115	111–109	1779–1890
	1 : .032	1 : 1.077	1 : 1.048	1 : 1.067	1 : 1.204	1 : 1.177	1 : .996	1 : 1.013	1 : 1.198	1 : .982	1 : 1.062
	1	2	3	4	5	6	7	8	9	10
D	163–151	226–238	141–153	139–125	206–227	204–209	200–297	142–177	193–187	188–196	1802–1960
	1 : .926	1 : 1.053	1 : 1.085	1 : .899	1 : 1.10	1 : 1.025	1 : 1.49	1 : 1.246	1 : .969	1 : 1.04	1 : 1.087
	1	2	3	4	5	6	7	8			
E	51–46	96–111	37–45	125–119	84–89	56–48	101–101	73–68			
	1 : .902	1 : 1.156	1 : 1.216	1 : .952	1 : 1.059	1 : .857	1 : 1	1 : .93			
	9	10	11	12	13	14	15				
E	88–92	101–112	86–78	88–86	71–94	63–84	53–62				1173–1235
	1 : 1.045	1 : 1.109	1 : .907	1 : .977	1 : 1.324	1 : 1.33	1 : 1.169				1 : 1.053
	1	3	4	5	6	7	8	9	10	11	
F	151–123	100–72	192–158	135–152	113–96	121–110	117–129	41–32	97–80	99–123	
	1 : .815	1 : .72	1 : .823	1 : 1.126	1 : .849	1 : .909	1 : 1.102	1 : .78	1 : .83	1 : 1.242	
	12	13	14	15	16	17	18	19	20	21	
F	95–123	154–153	44–33	68–96	209–202	59–57	169–189	70–73	55–52	51–43	2140–2096
	1 : 1.295	1 : .99	1 : .75	1 : 1.41	1 : .966	1 : .97	1 : 1.118	1 : 1.043	1 : .945	1 : .843	1 : .979
	1	2	4	7	9	10	12	13	15	16	18	19
G	64–64	103–97	51–72	25–27	28–38	39–26	59–79	36–34	42–27	79–83	33–39	34–22	
	1 : 1	1 : .94	1 : 1.41	1 : 1.08	1 : 1.36	1 : .67	1 : 1.34	1 : .94	1 : .64	1 : 1.05	1 : 1.18	1 : .647	
	20	21	22	23	24	25	26	27	28	30	31	
G	19–37	55–46	29–29	88–78	34–34	89–69	104–108	75–79	65–71	115–112	55–39	1321–1310
	1 : 1.95	1 : .84	1 : 1	1 : .89	1 : 1	1 : .78	1 : 1.04	1 : 1.05	1 : 1.09	1 : .974	1 : .709	1 : .9916

Grand Total.....9673–9951
1 : 1.0287

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TABLE X.

Showing Summary of the "High" Strain of the Warsaw Stock. Experiment III.

Generation.	1	2	3	4	5	6	7	8	9	10	Totals.
A						147 ♂s–187 ♀s (*1 : 1.272*)					
B	126–115 *1 : .913*	195–210 *1 : 1.08*	52–42 *1 : .81*	53–76 *1 : 1.43*	119–127 *1 : 1.07*	138–143 *1 : 1.04*	191–188 *1 : .98*	194–196 *1 : 1.01*	206–189 *1 : .92*	208–253 *1 : 1.22*	1482–1539 *1 : 1.039*
C	76–95 *1 : 1.25*	168–202 *1 : 1.20*	209–204 *1 : .98*	80–85 *1 : 1.06*	228–242 *1 : 1.06*	59–66 *1 : 1.12*	154–170 *1 : 1.10*	183–176 *1 : .962*	160–179 *1 : 1.12*	181–149 *1 : .82*	1498–1568 *1 : 1.047*
D	43–43 *1 : 1*	69–78 *1 : 1.13*	79–90 *1 : 1.14*	141–152 *1 : 1.08*	149–141 *1 : .946*	147–139 *1 : .946*	194–190 *1 : .979*	195–202 *1 : 1.04*	193–181 *1 : .94*	146–152 *1 : 1.04*	1356–1368 *1 : 1.009*
E	28–39 *1 : 1.39*	36–41 *1 : 1.14*	74–93 *1 : 1.26*	59–70 *1 : 1.19*	112–116 *1 : 1.04*	123–128 *1 : 1.04*	107–121 *1 : 1.13*	94–91 *1 : .97*			
E	92–105 *1 : 1.14*	52–62 *1 : 1.19*	84–82 *1 : .976*	67–121 *1 : 1.806*	105–126 *1 : 1.2*						1033–1195 *1 : 1.157*
F	145–146 *1 : 1.007*	214–245 *1 : 1.15*	249–246 *1 : .988*	28–65 *1 : 2.32*	168–179 *1 : 1.07*	169–162 *1 : .96*	154–162 *1 : 1.05*	77–63 *1 : .82*	133–146 *1 : 1.098*	79–74 *1 : .94*	

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TABLE X.—*Continued.*

	11	12	13	14	15	16	17	18	19	20	
F	100–102	214–228	195–226	182–199	177–149	208–214	194–207	121–135	148–157	93–86	
	1 : 1.02	*1 : 1.07*	*1 : 1.16*	*1 : 1.09*	*1 : .842*	*1 : 1.03*	*1 : 1.07*	*1 : 1.12*	*1 : 1.06*	*1 : .93*	

	21	22	23	24	25	26	27	28	
F	132–127	75–98	115–149	114–122	192–206	113–130	118–136	173–156	4080–4315
	1 : .96	*1 : 1.31*	*1 : 1.3*	*1 : 1.07*	*1 : 1.07*	*1 : 1.15*	*1 : 1.15*	*1 : .902*	*1 : 1.0576*

	1	2	3	4	5	6	7	9	10	11	12
G	57–69	58–56	85–91	49–56	33–27	84–72	26–46	44–49	90–94	112–122	92–83
	1 : 1.21	*1 : .97*	*1 : 1.07*	*1 : 1.14*	*1 : .82*	*1 : .86*	*1 : 1.77*	*1 : 1.11*	*1 : 1.04*	*1 : 1.09*	*1 : .90*

	13	14	15	16	17	18	19	20	21	22	23
G	65–67	46–51	128–109	73–75	53–49	73–86	41–44	62–49	34–32	68–76	63–98
	1 : 1.03	*1 : 1.11*	*1 : .85*	*1 : 1.03*	*1 : .93*	*1 : 1.18*	*1 : 1.08*	*1 : .79*	*1 : .94*	*1 : 1.12*	*1 : 1.56*

	24	25	26	27	28	29	30	31	32	33	34
G	36–27	39–60	80–69	53–33	52–62	134–108	42–35	50–48	49–39	103–113	71–87
	1 : .75	*1 : 1.54*	*1 : .86*	*1 : .62*	*1 : 1.19*	*1 : .81*	*1 : .83*	*1 : .96*	*1 : .796*	*1 : 1.097*	*1 : 1.23*

	35	36	37	38	39	40	41	42	43	44	45	
G	113–109	58–57	84–94	32–27	29–32	60–50	61–64	27–36	67–70	152–126	50–66	2878–2913
	1 : .97	*1 : .98*	*1 : 1.12*	*1 : .84*	*1 : 1.10*	*1 : .83*	*1 : 1.05*	*1 : 1.33*	*1 : 1.05*	*1 : .829*	*1 : 1.32*	*1 : 1.0122*

Grand Total..... 12,327–12,898
1 : 1.0463

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the opposite direction the "male" strain. This terminology will be used throughout this discussion. As the starting point of his female strain, he selected from nature a pair of flies which produced 52 males and 135 females and in his male strain he started with a pair which produced 84 males and 75 females.

Considering first the effect of selection upon his female strain we find here no conclusive evidence of any progressive change in the direction of the selection. (His data for this strain are given in Table XI.) To be sure, there is a slight increase in the relative number of females in his second and third generations over his first generation, but this fluctuation could easily be attributed to the small number of individuals examined. The greatest difficulty in assuming the effectiveness of selection here, is the fact that his last two generations are the ones in which he obtained the lowest relative number of females. Moenkhaus explains these low ratios of the last two generations in that he possibly made a poor selection in the preceding generation, but if we consider selection as having a cumulative effect (as Moenkhaus seems to consider it) it is difficult to see how one could lose by one poor selection all that he had accomplished in the previous selections. It must be admitted that by using as parents of each succeeding generation, pairs which threw the most extreme ratios in favor of the females, Moenkhaus was able to maintain a strain which on an average gave a relatively high number of females. But this was probably not due to any cumulative effect of selection but to the isolation of a peculiar type of female which will be discussed more fully later.

As to the male strain (Table XII)., Moenkhaus admits that the effect of his selection here has been slight and after examining his data carefully it is difficult to see how it could be assumed that there has been even a slight effect of selection. He started with a ratio (84 males to 75 females) which was only slightly different from normal and this slight difference was not transmitted to the first generation nor any succeeding one. So it seems that he had here a normal strain which would have given the same ratio regardless of the direction of the selection.

The female strain is the unusual one and is in need of an explanation. It throws some very exceptional ratios in favor of

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TABLE XI.

History of Moenkhaus's "Female" Strain.

206 (52 ♂s : 135 ♀s)

Generation.	1	2	3	4	5	6	7	8	9	10	11	Totals.
1	21–37	42–68	71–164	76–71	108–125	51–80	43–67	51–46	53–103	8–56	55–102	578–969 *1 : 1.67*
2						7 21–49	8 41–94	9 55–70	10 98–178			215–391 *1 : 1.82*
3				3 15–37	4 18–54	5 25–37	7 35–71					93–201 *1 : 2.17*
4	1 26–47	2 39–56	3 33–52	5 64–67	7 53–93	8 85–162	10 54–106					354–483 *1 : 1.36*
5					4 121–139	5 32–32	6 93–101	7 40–114	8 86–132			372–518 *1 : 1.39*
5	2 51–44	3 67–53	5 41–47	6 74–78	7 120–130	10 102–136	13 41–47					496–535 *1 : 1.07*

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TABLE XII.

History of Moenkhaus's "Male" Strain.

207 (84 ♂s : 75 ♀s)

Generation												Totals.
1	2	5	6	7	8	10	11					536–579
	79–68	99–121	71–88	67–73	60–57	72–71	86–100					*1 : 1.08*
2				3	5	7	9					220–223
				44–50	133–140	25–21	18–21					*1 : 1.00*
3	1	2	3	4	5	6	7	8	9			581–640
	71–98	115–101	76–84	51–73	42–37	45–48	36–39	61–52	84–108			*1 : 1.10*
4						1	5	6	7			142–147
						10–14	41–41	38–38	45–47			*1 : 1.04*
5	1	3	5	6	7	8	9	11				518–607
	40–32	45–144	46–35	26–26	146–144	72–98	64–74	79–84				*1 : 1.17*
6	1	2	4	5	7	8	9	10				561–654
	30–50	94–116	58–67	32–51	55–75	85–92	64–104	43–99				*1 : 1.42*
6	1	2	3	4	5	6	7	8	9	10	11	944–997
	118–109	99–107	164–198	141–157	61–85	56–63	24–27	116–109	42–40	73–67	50–35	*1 : 1.05*

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the females and these exceptional individual ratios caused the relative number of females in the female strain to be higher than normal. These unusual ratios can be readily explained by assuming that the female which Moenkhaus selected as the mother of his female strain carried a recessive sex-linked lethal factor and it will be pointed out later that all of Moenkhaus's data substantiate, very precisely, this assumption. The existence of such factors has been conclusively demonstrated by the recent work of Morgan and his students. A recessive lethal factor is any factor that brings about the death of the individual in which it occurs, provided its effect is not counteracted by the action of its normal allelomorph. Then if a lethal is sex-linked, all males which get it will die for they cannot carry its normal allelomorph since they possess but one X-chromosome. Since all males receiving the lethal factor die, this factor is never transmitted by the male and as a consequence the female can never be homozygous for it. Therefore the lethal factor has no effect on the female but she, by transmitting it to half of her sons, causes their death. Since half of the males die, a 2:1 ratio will result. A female carrying a lethal will transmit it to half of her daughters and they will always be heterozygous for it since they cannot receive it from their father.

Then the female which Moenkhaus used as the mother of his female line was probably a lethal female. It should be said that Moenkhaus is not to be criticised for not considering lethals in his paper, for lethals were not known till two years after his work was published. The original female gave a ratio which was an approximate 2:1 ratio. As mothers of the succeeding generations, Moenkhaus probably selected lethal bearing females (excluding female 5 of generation 4). These females should transmit the lethal factor to half their daughters and this expectation is realized, for from the 31 matings made in the female strain (excluding the offspring of female 5 of generation 4), fifteen 2:1 ratios resulted. Those considered 2:1 ratios are matings 3, 9 and 11 of generation 1; 7, 8 and 10 of generation 2; 3, 4 and 7 of generation 3; 1, 7, 8, and 10 of generation 4; and 7 and 8 of generation 5. So the number of 2:1 ratios obtained would justify the assumption of the presence of a lethal factor.

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Another indication of the presence of a lethal factor is the fact that in generation 4, when he used female 5 which gave a normal ratio, as mother of the next generation, all of her daughters also threw normal ratios. This female did not carry the lethal factor and none of her offspring showed abnormal ratios.

A third indication of a lethal factor is the fact that Moenkhaus found in crossing his male and female strains that the female is almost wholly responsible for the transmission of the sex-ratio. For he found if females from a strain possessing a high female ratio be mated with males from a strain possessing a low female ratio or vice versa, the offspring will show a sex-ratio which is wholly or very near that of the strain from which the females were taken. If his female strain carried a lethal factor and if his male strain was a normal strain, as it appears to be, the above result would be the expected one. For, if a lethal bearing female is mated to a normal male, she will transmit the lethal factor to half her daughters but a male from the lethal strain cannot transmit the lethal factor because all of the lethal bearing males die. Therefore the end result would be as Moenkhaus found, that the offspring will show sex-ratios like that of the strains from which the females came.

Of course, the only sure way of testing for a sex-linked lethal factor is to cross the suspected female to a male carrying another sex-linked factor and the resulting F_2 will be characterized by a deficiency in the class of normal males. So it is not possible to determine beyond doubt whether the unusual ratios in Moenkhaus's female strain were due to a lethal factor. But since several lethals have already been found and all of his data substantiate this assumption, it seems that this explanation is probably the correct one.

Discussion.

The writer's three attempts to modify by selection, the sex-ratio in the fruit fly have resulted in no clear-cut evidence that it was modifiable. In each experiment, the work covered more generations and many more individuals than the work of Moenkhaus. Since Moenkhaus's results could not be obtained in any one of the three experiments, it leads one to question Moenkhaus's conclusions or, at least, to question the general application

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of his findings. The fact that the difference which Moenkhaus found between his two strains can be readily explained to be due to a cause entirely independent of the cumulative effect of selection, makes his work support, rather than oppose the findings of the writer. It must be admitted that negative results in secletion work can never be conclusive, for the criticism may always be offered that selection over a greater period might have yielded different results. But, nevertheless, we feel justified in concluding from the data here presented that the sex-ratio in *Drosophila ampelophila* is not readily, if at all, modifiable by selection.

LETHALS.

Since the offspring of over 700 pairs of flies were examined for sex in these experiments, and since extreme ratios were sought, it would not be surprising to find a sex-linked lethal mutation. In fact, there is fairly good evidence that one such mutation occurred. This was mating number of 6 generation G of the low strain of the Bloomington stock (Table IV.). This gave an approximate 2:1 ratio (84 males to 180 females). Four matings were made from among the offspring of this mating and three out of the four gave 2 to 1 ratios while the fourth was doubtful. If a sex-linked lethal mutation had occurred, half of the four should have given 2 to 1 ratios and the other half normal. Since so few matings were made, it is possible that all the females chosen were lethal bearers. The stock was lost by accident at this time and further tests were not possible. But since the unusual ratio was transmitted it is probable that a mutation occurred here. In the high series of the Saratoga stock, there were several ratios which approximate a 2 to 1 ratio but none of the flies showing it transmitted this tendency to their offspring. Also in the high strain of the Warsaw stock, generation E mating 12 gave a 2 to 1 ratio. A large number of matings were made among the offspring of this pair but this unusual ratio was not transmitted to any of the progeny.

In all of the matings examined there were comparatively few ratios found which were two to one or more.[1] These extreme

[1] The unusual ratios more frequently occurred where the counts were very small. These cases were considered to have been due to some unfavorable environmental condition.

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ratios were more frequently in favor of the females and in only one case was there any evidence that a sex-linked lethal mutation had occurred.

In conclusion, I wish to express my thanks to Professor Fernandus Payne for the suggestion of this problem and also for many helpful suggestions and criticism while the work was in progress. I am also greatly indebted to my wife, Elmira Shierling Warren, for help, both in the carrying out of the experiment and in the preparation of the manuscript.

SUMMARY.

1. The sex-ratio in *Drosophila ampelophila* is 100 females to 95 males.

2. The age of the parent has no effect upon the sex-ratio of its offspring.

3. The difference which Moenkhaus found between his two strains, and which he attributed to selection, was probably due to the fact that his male strain was a normal one and his female strain was a lethal bearing one.

4. The sex-ratio in *Drosophila ampelophila* is not readily, if at all, modifiable by selection.

5. There was probably one sex-linked lethal mutation in the writer's selection stocks.

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EFFECTS OF AGING UPON GERM CELLS AND UPON EARLY DEVELOPMENT.

PART II., CHANGES IN MODERATELY AGED EGGS AND SPERM.

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In a previous publication (Goldfarb, '17), the writer determined the variation of freshly liberated sperm and eggs from freshly collected sea urchins of three different species. Two of these, *Toxopneustes* and *Hipponoë*, inhabit the shallow tropical waters of the Dry Tortugas, and the third, *Arbacia*, is common in the deeper colder waters near Woods Hole, Mass. In each species preliminary experiments had been made to ascertain the optimum conditions of development, such as volume and surface and filtration of sea water, concentration of eggs and sperm, etc., and such optimum conditions were used in all subsequent experimentation.

It was shown that even under these constant and optimum conditions, the freshly liberated eggs of different females varied in respect to (1) size, (2) jelly layer, (3) membrane formation, and (4) cleavage; that there was a surprisingly large variation, with respect to each of these four classes, in the eggs of different females; that the sperm of different males also varied, but to a much less degree. And finally the range of variation was determined for each species.

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It was suggested that these variations served as convenient and accurate indices of the physiologic condition or vitality of the freshly removed eggs or sperm of different individuals.

In the present paper the same technique, the same three species of sea urchin, the same nomenclature and the same precautions were taken. It is proposed to determine first the physiologic condition of the germ cells, at the time of liberation, from given individuals, and then to determine the nature and the extent of the changes in the eggs and sperm as they become increasingly "overripe," "aged" or" stale."[1] In the next study, Part III., it is proposed to study other changes that appear in very late stages of overripening or ageing of the germ cells. I reserve for Part III. a discussion of the entire problem, and the bibliography.

The changes that were observed in moderately aging eggs, and those which will be discussed in detail are: (1) size, (2) jelly layer, (3) membrane formation and (4) cleavage.

I wish to acknowledge my indebtedness to Dr. A. G. Mayer, Director of the Marine Biological Laboratory of the Carnegie Institution of Washington, and to Prof. F. R. Lillie, Director of the Marine Biological Laboratory at Woods Hole, Mass., for the opportunity of working on this problem at their respective laboratories, and for the many facilities offered in connection with this work.

Effect of Age Upon Size of Eggs.

Hipponoë and Arbacia.

The diameters of freshly removed unfertilized eggs of each freshly collected female, were measured with an ocular micrometer (no. 3 eye piece and 1/6 objective) and the usual precautions concerning pressure, focusing, sampling, etc., taken. At different intervals (ages) other samples of the eggs of the same female were measured. The data are brought together in Table I.

I have shown (Goldfarb, '17) that the normal size of freshly liberated eggs of *Hipponoë* varied from 18½ to 20 ocular units in diameter, with a mode of 19½ units. In the present series

[1] By age is meant the time since removal or extrusion of ripe germ cells.

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Table I.

Shows Change in Size and Loss of Jelly Layer with Age. Size is Given in Ocular Micrometer Units of the Diameter of the Eggs at Different Ages. *Hipponoë.*

No.	Date.	Age of Eggs, Hrs.	Diameter of Eggs in Ocular Micrometer Units. Number of Eggs.									Per Cent. of Eggs with Jelly Layer.	Per Cent. Loss of Jelly per Hour.
			21.0.	20.5.	20.0.	19.5.	19.0.	18.5.	18.0.	17.5.	17.0.		
1	6/15	5				*	*						
		22	*	*	*								
2	6/23	5				1	8	1				97	
		29				4	7	1				78	0.7
3	6/28	½				1	7	4				100	
		18½				8	18	3				100	0.0
4	6/28	½			2	5	6	2				100	
		18½			3	8	5	0				94	0.3
5	6/25a	2			2	7	1					100	
		5		2	8	3	1					91	
		24		7	1	3	2					40	2.7
6	6/27a	3			1	12	3					96	
		9			12	5	1					96	
		27		1	9	2	0					79	0.7
		33		0	13	3	0						
7	6/26a	0		1	3	6	3					86	
		18		3	3	6	0					77	
		24				9	5	1				62	1.0
		40						1	9	6	5	0	
		47							1	9	2	0	
8	6/26b	0			8	3						95	
		18	2	4	8	4						95	
		24			7	3						72	0.9
9	6/26c	0				0	4	8				90	
		18				0	15	2				60	
		24				3	8	3				6	3.5
		40				3	12	1				2	
		47				1	6	6				1	
10	6/25b	2				1	9	1				63	
		5				1	10	1				1	
		24					5	7	4	1		0	2.8
11	6/27b	3	2	4	3	6	0	0				80	
		9			1	4	9	1				10	
		27			1	9	2	0				1	3.3
		33			1	2	3	0	2	2	1	0	
12	6/26c	0			5	10						99	
		18		2	12	2						98	
		24		0	12	1	1					98	0.4
		40		1	11	1	1					62	
		47		1	12	1	0					17	
Variation in freshly liberated eggs of different females...			2	6	34	72	54	16	0	0	0	90	

the same variations occurred in the freshly liberated germ cells. As the eggs aged, there was a clear, considerable and progressive change in size. The observations may best be considered in three groups.

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Female 1, is an example of the first group. When her eggs were 5 hours old, *i. e.*, 5 hours after liberation, their diameters were 19 and 19.5 units. When her eggs were 22 hours old, they measured 20, 20.5 and 21 units, *a clear and sharp increase in minimum, mode and maximum diameters, with age.*

In female 2, observations were made when the eggs were 5 and 29 hours old. In this female there was also an enlargement but very small. In females 3 and 4, observations were made at ½ and 18 hours respectively. In each female the eggs enlarged with age. In female 5, three observations were made, viz., 2, 5 and 24 hours, with a progressive enlargement at each successive age. In female 6, observations were made at four intervals, 3, 9, 27 and 33 hours. As in female 5, there was at first a rapid enlargement of the eggs, then a slow increase, and, in very late aging, a rapid increase again. The average diameters at each of the four intervals was 19.44, 19.80, 19.77 and 19.96.

In all the eggs of this first group, there was a cumulative increase in volume, with age, for the first 33 hours.

The second group includes such females whose eggs were observed over a longer period than 33 hours. In this group as in the first, the eggs also enlarged at first, with age, but after a longer interval there occurred a secondary diminution in size, and in very old eggs became even smaller than the norm.

For example, the eggs of female 7 were measured at 0, 18, 24, 40 and 47 hours. When 18 hours old the eggs were slightly larger than at the first observation. At 24 hours, the eggs were clearly smaller, and at 40 hours smaller still. At 47 hours they were much below their original size and below the norm for the species. The maximum size had diminished from 20.5 to 18, the mode from 19.5 to 17.5, the minimum from 19 to 17.

In female 8, there was the same initial increase and subsequent diminution towards the norm, when 24 hours old. In female 9, observations were made at 0, 18, 24, 40 and 47 hours. There occurred the usual enlargement until the 40th hour, after which there was a definite diminution in volume.

In a third group, of a few females only, the eggs though freshly removed, were in poor physiologic condition. In this group *the initial enlargement did not take place.* In these eggs

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there occurred a direct diminution with age. In this group belong females 10 and 11, and probably female 7.

It should be recalled that the physiologic condition of any set of eggs was not determined merely by egg size, but by the results of several tests, such as the jelly layer test, the membrane test as well as the cleavage test, etc. By all of these tests, it was clearly and definitely shown *that eggs in good physiologic condition at the time of liberation, increased steadily with age, within the limits shown in Table I., and subsequently diminished in size by a process of fragmentation described in Part III. Eggs in physiologically*

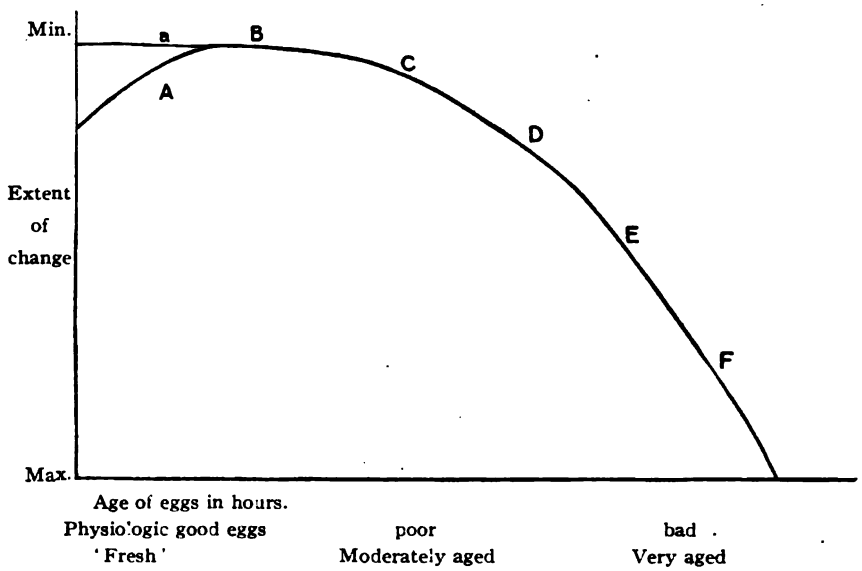

FIG. 1. Extent of morphologic and physiologic change in aging or deteriorating eggs. *A'*, *B*, *C*, *D*, *E*, *F*, represents the change in volume, rate of membrane formation, rate of cleavage, total cleavage (*Hipponoë*). *A*, *B*, *C*, *D*, *E*, *F*, represents the change in width of fertilization membrane, loss of jelly, total cleavage, etc. *A'*, *B* is period of superripening; *B*, *C*, *D*, the period of overripening or deterioration; *D*, *E*, *F*, the period of extreme deterioration or dying of the eggs.

poor condition at the time of liberation behaved like half-aged eggs, that is, they either enlarged but little, or did not enlarge at all, and subsequently diminished to and below the norm of the species.[1]

Figure 1 represents graphically and schematically the change

[1] So constant is the relation between volume and physiologic condition, tha one can predict the one from the other, with remarkable exactness.

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in volume with age. Physiologically good eggs show an initial rapid increment, region *A* of the curve; then a progressively slower increment, region *B;* thirdly a progressive *diminution*, region *C*, towards *D* or even below the norm of the species to *E*. Eggs in moderately poor physiologic condition at liberation have already undergone physiologic deterioration, corresponding with part *A* within the body of the mother. Upon liberation they show only parts *B*, *C*, *D*, etc., of the curve. Eggs in very poor condition upon liberation have undergone changes represented by parts *A* and *B*, within the body of the mother, and upon liberation show only parts *C* and *D*, etc.

The significance of this increment with age lies in the changed permeability of the cortical layer which is denoted by increase in volume. This progressive increase in permeability permits increasing volumes of sea water to penetrate the egg and thus enlarge it. This process continues until partial cytolysis (fragmentation) reduces the size once again towards the norm, or until complete cytolysis destroyed the eggs altogether.

The change in size with age really measures the change in permeability of the cortical layer.

Similar observations were made upon *Arbacia* eggs, and the changes are so much in agreement with those of *Hipponoë* that it seems unnecessary to describe them here. There is the same initial increment followed by the secondary diminution with age. And the rate of change depended upon the physiologic condition of the eggs at the time of liberation and upon temperature. The two species differ in the markedly slower rate of change of *Arbacia*, due in largest part to the lower temperature of the sea water.

Effect of Age Upon the Jelly Layer.

Hipponoë and Arbacia.

The jelly layer of the same sea-urchin eggs were made clearly visible by adding Chinese black to the sea water, as suggested by F. R. Lillie. Samples of 150 to 200 consecutive freshly removed (and unfertilized) eggs of each female were examined, avoiding mechanical or chemical removal of the jelly, and the presence or absence of the jelly layer recorded. At successive intervals

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other samples of the same female were examined, and the results reduced to percentages as recorded in Table I.

I have shown (Goldfarb, '17) that the freshly liberated eggs of different females vary considerably in the per cent. possessing the jelly layer. In *Hipponoë* the per cents vary from 100 to 63 per cent., in *Arbacia* from 100 to 59 per cent. These differences, I suggested, indicated corresponding differences in physiologic condition of the eggs at the time of liberation.

Whatever the condition of the eggs at the time of liberation, with increasing age there was a continuous decrease in the number of eggs with the jelly layer present.

The data may best be considered in two groups, according to the physiologic condition of the eggs at the time of the first observation.

In the first group are those females whose eggs were in good physiologic condition. This was indicated by the high per cent. (*i. e.*, above 95 per cent. with jelly layers) at the first observation, as well as by other tests. In this group are placed females 2, 3, 4, 5, 6, 8 and 12, and the corresponding percents of jelly layers was 97, 100, 100, 100, 96, 95 and 99 respectively.

In the second group are placed those females whose eggs were in poor physiologic condition. In this group are included females 7, 9, 10 and 11, whose corresponding jelly counts were, 86, 90, 63 and 80 per cent., respectively.

In both groups the surrounding jelly layer progressively disappeared with age, as seen in Table I. But the rate of loss was different. An example of the first group is female 8. When 0 hours old, 95 per cent. possessed the jelly layer, when 18 hours old 95 per cent., and when 24 hours old only 72 per cent. retained the layer. Female 11 is an example of the second group in which the loss was decidedly greater. The eggs when 3 hours old showed 80 per cent. with jelly layers, when 9 hours old but 10 per cent., when 27 hours old only 1 per cent., and when 33 hours old none of the eggs possessed the jelly envelope.

The two groups differ then, not only in original per cent. with jelly layers, but also in the rate of loss of this layer. In the first, containing physiologically good eggs, the loss of the jelly layer per hour, for the indicated intervals for the different females, was 0.7, 0.0,

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0.3, 2.7, 0.7, 0.9, 0.4 per cent., with an average of 0.81 per cent. loss per hour. In the second group with physiologically poor eggs the loss was, 1.0, 3.5, 2.8, 3.3 per cent. per hour with an average of 2.65 per cent. or over three times as rapid. I cannot account for the only apparent exception, namely female 5, whose rate of loss was 2.7 per cent.

Fig. 1 represents the loss of jelly with age. With physiologically good eggs, the numbers with jelly is a maximum or nearly so, and the rate of loss with age is small, part *a* and *B*. As the eggs become physiologically poor, and in eggs which were in poor physiologic condition at the time of liberation, the numbers with jelly is much less, and the rate of loss with age is more rapid, parts *C*, *D*, etc. It should be noted in passing that parts *B*, *C*, *D* and *E*, are parallel with the corresponding parts of the curve showing change in the volume of eggs with age.

The observations upon *Arbacia* eggs are in entire accord with those of *Hipponoë*. They differ only in the slower rate of loss. One example may illustrate this slower rate. The eggs when freshly removed showed 90 per cent. with their jelly layers. When 41 hours old, 50 per cent. possessed the layer, when 46 hours, 19 per cent., when 70 hours, 1 per cent., and when 72 hours none of the eggs possessed the jelly layer. *In both species of eggs the loss of jelly was a function of age, and physiologic condition at time of the first observation.* If the loss of jelly is known, one can predict (provided proper precautions are taken) the physiologic condition of the eggs and vice versa, from the physiologic condition one may determine the loss of jelly.

Loeb, Harvey and F. R. Lillie in particular, as well as others, have recorded the loss of jelly in old eggs. My observations show not only a loss with age, which is different for different species, but I have determined the rate of loss with age. More than this, I have shown that the rate of loss depends not only upon the species and temperature, but upon the condition of the eggs at liberation, and finally that loss of jelly serves as another symptom of the physiologic condition or vitality of the eggs at any time, and that this vitality diminished at a known rate with age.

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Effect of Age upon Membrane Formation.

A third sympton of aging and physiologic deterioration is the change in fertilization membrane. A number of investigators have observed that old eggs do not form fertilization membranes (Hertwig, R. and O., '86, Loeb, '03, '15, Harvey, '10, '14, F. R. Lillie, '14, etc.). Harvey ascertained the exact age in *Arbacia* beyond which, namely 52 hours, no membranes were formed. Just, '15, observed that the jelly layer, which follows fertilization in *Nereis* was retarded with aging.

Before considering in detail the changes in the fertilization membrane, with age, and the causes of such changes, it should be recalled that freshly liberated eggs of freshly collected females, when fertilized under constant and optimum conditions, varied considerably in the rate of membrane formation. Such freshly liberated eggs may be placed into three groups according to their physiologic condition, namely (1) those that form membranes within 2 minutes after fertilization, which are in good physiologic condition. (2) Those that form membranes in 3 to 7 minutes, and which are in moderately poor condition, and (3) those that do not form membranes, which are in poor physiologic condition. It should also be recalled that the rate of membrane formation is further complicated by the differential effect of eggs and sperm. I will mention but two of the experiments, which are typical, to illustrate this differential effect. In experiment 1, Table II., *the eggs of one female were fertilized by the sperm of 5 different males.* All the germ cells were 43 minutes old. Four of the males induced membrane formation in 1½ minutes, the fifth in 2 minutes. Even greater uniformity occurred when the germ cells were 1 hour old, for *the eggs of all 5 samples of the same female formed their membranes at the same time, namely 1 minute.* When the germ cells were 1½ and 2¼ hours old, the eggs again reacted in the same manner for none formed membranes.

In contrast to this uniformity of behavior, when the eggs of one female were fertilized by different males, are those experiments in which the eggs of different females were fertilized by the one male. For example, in experiment 2, the eggs of 3 females were

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fertilized by the one male, when the germ cells were 20 minutes old. Fertilization membranes first appeared in the three groups of eggs as follows, 3½, 1½ and 1¾ minutes respectively. When the germ cells were 80 minutes old, they appeared in 2⅔, 1⅔, 1 5/6 minutes; when 140 minutes old, 1⅔, 1½ and 1 minute; when 280 minutes, ⅔, 1¼ and 1 minute.

In the other experiments similar results were obtained, i. e., uniform or nearly uniform rate of membane formation, when the eggs of one female were fertilized by different sperm, and frequently,

TABLE II.*a*.

SHOWS CHANGE IN RATE OF MEMBRANE FORMATION WITH AGE, OF EGGS OF DIFFERENT FEMALES. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.	Minutes Required Before first Membranes Appeared.						Average No. Minutes.
			Male 1.	2.	3.	4.	5.	6.	
1	7/3	43	2	1½	1½	1½	1½		1.6
		59	1	1	1	1	1		1.0
		89	N	N	N	N	N		N
		140	N	N	N	N	N		N
			Female 1.	2.	3.	4.	5.		
2	7/14	20	3½	1½	1¾				2.25
		80	2⅔	1⅔	1 5/6				2.05
		140	1⅔	1½	1				1.39
		210	1¼	1¼	1¼				1.25
		280	⅔	1¼	1				0.97
		370–600	N	N	N				N
3	7/21	120	1½	1					1.25
		323	1⅓	1					1.15
		450	N	N					N
4	7/16	160	N	N	2½	2½	0	2½	2.5
		300	N	N	N	N	N	1½	0.5
		500–600	N	N	N	N	N	N	N
5	7/7	75	2	3	2½	3½			2.75
		84	1	2	1½	2			1.62
		180	2	2	4½	6			3.62
6	7/12	17	1⅔	1	1				1.22
		42	1½	1	1				1.16
		99	½	¾	¾				0.66
		161	⅓	1	⅔				0.66
		214	¼	⅔	¾				0.53
		294	2½	⅝	N				1.11
		360	5½	N	N				1.83
		420	N	N	N				N
7	7/19	130	3	3	2	1½	1½	1½	2.08
		240	N	2	2	1⅓	1⅓	1½	1.36
		350	N	2	2	2	2	2	1.83
		470	N	3½	4	N	2½	2½	2.08

N = no membrane in 10 minutes.

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wide differences when the eggs of different females were fertilized by the one male. There was a far greater variation among the eggs of different females than among the sperm of different males, or, putting the matter in a slightly different way, the extent of variation in development is conditioned much more by the eggs than by the sperm.

With these facts in mind, we may proceed with the examination of the effect of age upon the membrane formation in physiologically "good," "moderately poor" and "poor" eggs. The data are brought together in Tables II.*a* and II.*b*.

TABLE II.*b*.

SHOWS CHANGE IN RATE OF MEMBRANE FORMATION OF AGEING EGGS AND SPERM.

Arbacia.

No.	Date.	Age of Germ Cells in Hours.		Minutes Required Before Membranes Appeared.						Average No. Minutes.
		♀.	♂.	Female 1.	2.	3.	4.	5.	6.	
1	8/12	4	4	2	1½	3	2	2		2.1
		23	23	3	7	N	N	N		5.0
		28–48	28–48	N	N	N	N	N		N
2	8/14	4	4	2	2	2	2	2	2	2.0
		24	1	N	N	3½	2	N	N	2.7
		31	8	N	N	N	N	N	N	N
		49	1¼	N	N	N	N	N	N	N
		5	5	1½	1½	½	½	½		
3	8/16	1½	1½	1½	1½	1½	1½	1½	1½	1.5
		18½	18½	3½	N	3½	3½	3½	N	3.5
		23	23	N	N	N	N	N	N	N
		24	4	2½	N	2½	N	6	N	3.6
		42	1	N	N	N	N	N	N	N
		48–75	1/10–¾	N	N	N	N	N	N	N
4	8/18	1	23	7	3	3	3			
5	8/19	1	45½	2	N	2½				
6	8/20	5	73	2½	4	4½	5			
		10	95	2½	N	N	N			

N = no membranes in the first 10 minutes.

In the first group, *containing physiologically good eggs, the rate of membrane formation was accelerated with age.* For example in experiment 2, the eggs of the 3 females were fertilized by one male when the germ cells were 20, 80 140, 210, 280, 370, 440 and 550 minutes old. The eggs of female number 1, for instance, formed their membranes at the above ages in 3½, 2⅔, 1⅔, 1¼ and ⅔ minutes, and none after 370 minutes. The eggs of female number 3 formed their membranes in 1¾, 1⅚, 1,

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1¼ and 1 minute and none after 370 minutes. The average for all 3 females at the give ages was 2.25, 2.05, 1.39, 1.25, 0.97 minutes, and none between 370 and 600 minutes when the experiment terminated. A corresponding acceleration in the rate of membrane formation, with age, occurred in all the other experiments with physiologically good eggs.

In the second group of physiologically poor eggs, there was also an acceleration in rate of membrane formation with age, but subsequent to this, there was a period of retardation, and ultimate inability to form membranes.

For example in Experiment 5, the eggs of 4 females were fertilized by the same male when the germ cells were 75, 84 and 180 minutes old. The eggs of female no. 1 formed their membranes at these ages in 2, 1 and 2 minutes respectively, in female 3, in 2½, 1½ and 4½ minutes, in female 4 in 3½, 2 and 6 minutes respectively. Female 2 alone did not show any retardation. The average for all 4 females was *2.75, 1.62 and 3.62 minutes respectively.*

In experiment 6, of the three females whose eggs showed an initial acceleration, only one showed a secondary retardation. The observations in this experiment were made at more intermediate ages namely, 17, 42, 99, 161, 214, 294, 360, 420 and 430 minutes respectively. The eggs of female no. 1 formed membranes in 1 ⅔, 1½, ½, ⅓, $\frac{1}{5}$ minutes and formed no more membranes after 420 minutes. The average rate for all three females for the corresponding ages, was *1.22, 1.16, 0.66, 0.53, 1.11 and 1.83 minutes* respectively, and none after 420 minutes.

In experiment 7, 5 out of 6 females showed the initial acceleration with age, and the secondary retardation. The average for the 6 females for the following ages 130, 240, 350 and 470 minutes, was *2.08, 1.36, 1.83 and 2.08 minutes respectively.*

The results may be graphically represented as in Fig. 1. The ordinates represent the inverse time or rate of membrane formation. The abcissas represent age of germ cells. With physiologically good eggs, the time and rate of membrane formation decreases with age, parts *A* and *B* of the curve. Beyond certain limits when the eggs are in poor physiologic condition, the time and rate is correspondingly increased, parts *C*, *D*, *E*,

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etc. In eggs, physiologically poor at the time of liberation, there is either a short period of acceleration, part *B*, followed by a long period of retardation, parts *C*, *D*, *E*, etc. In eggs in still poorer condition, the retardation takes place from the very beginning of the experiment, parts *C*, *D*, *E*, etc. Hence the better the condition of the eggs the longer the membrane-forming period and the longer the period of acceleration. The poorer the condition the shorter the periods.

I have chosen the reciprocal curve, for in this form it becomes quite clear that it is identical with Fig. 1, showing increase in size of eggs, and decreased per cent. of jelly layers, with age. I will discuss this correlation later.

There is one other matter that deserves brief mention. It was observed repeatedly that physiologically good eggs form membranes a considerable distance from the egg surface. With increasing age and with change in rate of membrane formation, the membranes are formed closer and closer to the egg, until they could barely be distinguished from the plasma membrane, and finally did not appear at all. With this reduction in width of the perivitelline space there appeared to be a greater tendency toward scalloping or blistering in the early appearance of the membrane. The Hertwigs in '86 observed a similar narrow perivitelline space and scalloping in ageing eggs.

Arbacia.

Some observations upon *Arbacia* eggs gave similar results, with one exception, namely, there was no initial acceleration of rate in membrane formation so characteristic of *Toxopneustes*. But this lack of acceleration may however have been due to insufficient early observations. The facts are brought together in Table II.*b*.

In experiment 1 (Table II.*b*), the eggs of 2 out of the 5 females that formed membranes at all, after the first observations, showed retardation in rate with age.

In experiment 3, the eggs of 4 out of the 7 females that formed membranes after the first observation, also showed retarded rate of membrane formation.

In experiment 2, one female showed retardation, another

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showed no change in rate and the other 4 did not form membranes after the first observation.

At this point the question arose whether the inability to form membranes was due to the sperm or to the egg or to both. The matter was tested as follows: *after eggs and sperm of the same age no longer formed membranes*, the eggs were fertilized by fresh sperm, and vice versa, freshly liberated eggs were tested with old sperm. In the first case, *i. e.*, stale eggs fertilized by fresh sperm, fertilization membranes reappeared, and were formed in most instances as rapidly as in previous matings. For example, in experiment 3, membranes ceased to appear when both germ cells were 23 hours old. When 24 hours old, the eggs were fertilized by 4-hour-old sperm, and membranes appeared in 3 out of 7 females. The eggs of 2 females formed membranes in 2½ minutes each, which was more rapid than when the eggs were 18½ hours old. The third formed membranes in 6 minutes. These observations showed that the first inability to form membranes was not due to the absence of membrane-forming substance in the eggs.

When fresh sperm was used with eggs 42, 48, 65 and 70 hours old, no membranes were formed. This showed that at this age the lack of membranes was now due to a change in the eggs which began about 24 hours after liberation in some females, and in 42 hours in other females. The membrane substance was gone.

In the reverse experiments, when increasingly old sperm was used to fertilize fresh eggs, membranes were formed as rapidly and as extended from the surface of the egg as when fresh sperm was used (within certain limitations). For example in experiment 4, 23-hour-old sperm caused membranes to appear in 7, 3, 3, 3 minutes in the 4 females. In experiment 5 the sperm which was 45½ hours old formed membranes in 2 of the 3 females in 2 and 2½ minutes respectively. In experiment 6, when sperm was 73 hours old, membranes appeared in 2½, 4, 4½ and 5 minutes respectively. Even when sperm was 95 hours old (experiment 6) one out of 4 females formed membranes in 2½ minutes.

These and other facts clearly show that *the rate and charac-*

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ter of membrane formation, in aging eggs, are functions largely of the egg and determined by their physiologic condition at liberation and by their subsequent age. Physiologically good eggs form clear membranes rapidly with either fresh or old sperm (the maximum age of such sperm was not ascertained). Physiologically poor eggs, form membranes more slowly, closer to the egg surface or do not form membranes at all with either fresh or stale sperm. The change in rate of membrane formation with age, constitutes the third index of the physiologic deterioration of the eggs with age.

Effect of Age Upon Cleavage.

Synchronous Aging of Germ Cells.

Aging or physiologic deterioration may be measured either (1) by a change in size, or (2) a loss of the jelly layer, or (3) a change in rate and character of membrane formation. There is another, more exact and more finely graded index of the changes in the germ cell with age, namely, (4) changes in cleavage.

Hertwig, '96, and Loeb suggested that with age there was a decreasing cleavage. F. R. Lillie, '14, showed in much greater detail that there was a decrease and indicated the extent of the decrease.

In Study I., I showed that under constant and optimum conditions freshly liberated eggs from different freshly collected females frequently differed widely in the total per cent. of cleavage in a given time, even when fertilized by the same male. In *Toxopneustes* the different females vary from 11 to 87 per cent., in *Hipponoë* from 5 to 81 per cent., in *Arbacia* from 0 to 90 per cent. Such differences were ascribed to differences in physiologic condition of the eggs of the different females at the time of liberation.

In preliminary experiments, a given pair of tested sea urchins were used for successive fertilizations. From 100 to 200 of the fertilized eggs were examined at each interval, and the sperm used was freshly prepared from the dry sperm.

In one group of experiments there was an unmistakeable direct reduction in the total cleavage with age. For example, in experiment 1, the eggs of 3 females were fertilized by one male when

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the germ cells were from ¼ to 6 hours old. The per cent. of the total number of eggs that cleaved at each interval was 98 per cent. when 17 minutes old, 80 per cent. when 42 minutes old, 70 per cent. when 98 minutes old, 33 per cent. when 161 minutes old, 40 per cent. when 214 minutes old, 18 per cent. when 294 minutes old, 0 per cent. when 360 minutes old. The other 2 females showed corresponding decreases. The average for the 3 females was 94, 62, 54, 28, 31, 11 and 0 per cent. respectively. See Table III.

In the other group of experiments, there was an initial increased cleavage in the eggs of some or all of the females, followed by a definite and progressive decrease. For example in experiment 6, the eggs of female 1 and 4 decreased in cleavage with age, as in the first group. But the eggs of females 2 and 3 increased in cleavage for a brief period after the first observation, and then the per cent. decreased. This appeared to me at the time to be an error in observation, but similar increments occurred in other experiments. In experiment 7 the eggs were fertilized at early and frequent intervals, namely, 7, 20, 43, 59, 89 and 140 minutes. All 5 series of mating showed an early *progressive increased cleavage*, followed after 1 hour, by a decrease. The average for all 5 series was 49, 66, 88, 62, 40 and 25 per cent.

It is probable then that in the other experiments the apparent absence of an initial increased cleavage was due to failure to make sufficiently early and sufficiently frequent intervals, or possibly that the increase in cleavage becomes evident only in eggs in poor physiologic condition.

In *Arbacia* only the direct decreased cleavage was observed. Whether an initial increase occurred I cannot say. In the first place insufficient early observations were made, and in the second place, with physiologically good eggs, the maximum or nearly maximum cleavage took place, so that any physiologic change could not be manifested by an increased cleavage.

Only two experiments are given to illustrate the behavior of aging eggs of *Arbacia*. In experiment 8, the eggs of five females fertilized by one male, averaged 80 per cent. when 4 hours old, 33 per cent. when 23 hours old, 0 per cent. when 28½ hours old. (See Table III.)

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Table III.

Shows Change in Total Cleavage with Progressive Age of Germ Cells. *Toxopneustes* and *Arbacia*..

Toxopnuestes.

No.	Date.	Age of Germ Cells in Minutes.	Per Cent. of Eggs that Cleaved. Females 1.	2.	3.	4.	5.	6.	7.	Time After Fertilization, Minutes.	Average Per Cent. Cleavage.	Per Cent. Loss per Hr.
1	7/12	17	98	98	87					120	94	
		42	80	76	31						62	
		99	70	72	20						54	
		161	33	38	14						28	
		214	40	30	23						31	
		294	18	14	2						11	
		360	0	0	0						0	18.0
2	7/19	130	82[1]	96	83	96	98	94		120	91	
		240	93	99	84	89	93	91			91	
		350	92	98	82	64	81	12			71	
		470	77	64	70	4	82	10			51	7.0
3	7/16	160	50	74	81	96	18[1]	14	40	120	53	
		300	34	61	37	92	34	0	50		40	
		500	28	8	20	29	1	6	25		16	6.5
4	7/21	120	51	98						120	74	
		323	11	84							47	8.0
		450	0	0							0	
5	7/14	20	92	47	98					120	79	
		80	99	95	97						97	
		140	98	95	97						96	
		210	96	93	97						95	
		280	92	82	87						87	
		370	62	69	85						72	
		440	82	59	87						76	
		500	43	10	36						29	8.2
		810	0	0	0						0	
6	7/7	75	91	41	67	86				120	71	
		84	75	87	98	80					85	
		180	80	80	17	60					59	7.2
7	7/3	7	80	23	35	68	43			113	49	
		20	95	46	75	73	42			105	66	
		43	80	85	88	95	95			87	88	
		59	62	71	76	19	86			91	62	
		89	38	50	73	31	11			91	40	
		140	34	12	9	11	11			85	25	

Arbacia.

8	8/12	4 hrs.	95	97	18	95	95			60	80	
		23 "	57	49	0	0	60			67	33	2.5
		28½ "	0	0	0	0	0			60	0	
9	8/16	1½ "	100	98	100	88	98	92	100	64	96	
		18½ "	75	20	95	62	88	55	51	60	63	1.8
		23 "	0	0	0	0	0	0	0	60	0	

[1] Probably an error in recording.

Fig. 1 represents schematically the change in rate of cleavage with age.

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The per cent. cleavage is a convenient and finely graded index not only of the physiologic condition of the eggs at the time of liberation, but at subsequent intervals. It became then a simple matter to compute the rate of deterioration with age for a given female, or group of females. The rate of deterioration, *i. e.*, the reduction in cleavage, in per cent. per hour, varied from 6.5 per cent. per hour in experiment 3, to 18.0 per cent. in experiment 1, viz: 6.5, 7.0, 7.2, 8.0, 8.2 and 18 per cent. per hour, for nearly comparable periods. These rates are strikingly greater in *Toxopneustes* (from tropical waters) than in *Arbacia* (of the temperate waters of Massachusetts). Experiments 8 and 9 are illustrative of the rate of deterioration in *Arbacia*, viz: 1.8 per cent. per hour, in experiment 9, 2.5 per cent. per hour, experiment 8. When it is recalled that the difference in temperature of the waters at the two laboratories is about 10° C., and the difference in physiologic deterioration 3 to 10 times as great, it must be evident that the difference in rate is conditioned not only by temperature but by differences in HO concentration, and the egg protoplasm of the different species.

The above data did not make clear whether the decreased cleavage was due to the deterioration of the eggs or of the sperm or both sperm and eggs. The answer to these questions could be obtained in two ways: First to fertilize increasingly old eggs each time with freshly liberated and tested sperm, which would test the rate and degree of degeneration of the eggs. Secondly to fertilize freshly liberated and physiologically good eggs with fresh suspensions of increasingly old sperm, and test the degeneration of the sperm. Both series of experiments were made.

Effect of Age Upon Eggs.

Aging Eggs fertilized by Fresh Sperm. Longevity of Eggs. Toxopneustes.

In the following experiments, eggs and sperm aged synchronously until the eggs either no longer cleaved or only a very small per cent. cleaved. Then samples of the same eggs were fertilized at each subsequent interval by freshly liberated sperm. See Table IV.

In experiment 1, for example, the eggs of 4 females fertilized

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Table IV.

Shows Change in Cleavage when Progressively Aging Eggs Are Fertilized by Sperm of Varying Ages. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.		Per Cent. of Cleavage Eggs.						Time in Minutes.	Average Cleavage Per Cent.
		♀.	♂.	Female 1.	2.	3.	4.	5.	6.		
1	7/2	64	64	0	1	0	1			120	½
		209	19	8	85	70	10				43
		250	60	24	80	50	26				45
		309	120	3	44	14	17?				19
		400	3	8	70	2	10				22
2	7/12	420	420	6	2	0				120	3
		436	60	98	92	60					83
3	7/14	500	500	43	10	36				120	29
		600	2	50	36	81					55
		720	720	0	0	0					0
		10	600	33	0	0	10				10
4	7/19a	660	660	0	0	0	0	0	0	120	0
		660	90	95	94	13	32				59
		675	105	10	50	10	12	10	7		16
5	7/19b										
	a)	610	610	0	4					120	
				0	0						
				0	4						1
	b)	640	60	80	8						
				36	50						
				70	25						45
	c)	20	600	47	33	4	23				25
	d)	30	30	18	44	22					28
6	7/16	660	660	5	5					120	5
		7	660					91			91
		670	7	69	54	78					
				78	78						
				40							
		7	7				100				100
7	6/21	660	660	0						120	0
		660	20	18							18
		660	20	13							13
		20	660	0	0						0
		20	20	0	0						0

by the one male, gave a decreasing per cent. of cleavage with age, until the germ cells were 64 minutes old, when only 0, 1, 0 and 1 per cent. of the eggs of each of the females cleaved. The eggs were allowed to age further until they were 209 minutes old, when they were fertilized by freshly liberated sperm, and cleavage rose to 8, 85, 70 and 10 per cent. respectively. This was 42½ per cent. greater than the previous observation. It was clear that the failure to cleave when 64 minutes old was due to the almost complete deterioration of the sperm. It is not clear from this experiment how much the eggs have deteriorated. For

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as I have shown the results obtained by sperm from different individuals may not be compared without further testing. In this experiment, when the eggs were 225 minutes old, and sperm only 35 minutes old, 32 per cent. cleaved. When the eggs were 250 minutes old and the sperm 60 minutes old, the average cleavage was 45 per cent. Even when the eggs were 400 minutes old, and fertilized by freshly liberated sperm, 22 per cent. cleaved. Hence it is clear that the decline to the zero mark when both germ cells were 64 minutes old, was due not to the death or incompatibility of the eggs, but of the sperm.

Even more striking results were obtained in other experiments. In experiment 2, for example, cleavage decreased progressively until the germ cells were 420 minutes old, and 6, 2 and 0 per cent. of the eggs cleaved. When the eggs 16 minutes later, *i. e.*, 7¼ hours old, were fertilized by sperm only 1 hour old, the cleavage was 98, 92 and 60 per cent. or an increase of 80 per cent. over the last reading. When the eggs of these same females were liberated, and immediately fertilized they averaged 80 per cent. hence it is probable that very little real deterioration of the eggs occurred during the first 7 hours after liberation.

In experiment 3, when the germ cells were both 8 hours old, the average cleavage was 29 per cent., when 12 hours old 0 per cent. When the eggs were 10 hours old and the sperm 2 minutes old, the average cleavage rose to 55 per cent.

In experiment 4, 11-hour-old germ cells gave 10 per cent. cleavage. Eleven-hour-old eggs by moderately fresh sperm gave 59 per cent. cleavage. Fifteen minutes later using the same eggs and sperm, only 16 per cent. cleaved. The marked reduction was due again to the rapid deterioration of the sperm of the second male.

In experiment 5, the eggs of 2 females were tested by 3 different males. When the germ cells were 5 hours old 1 per cent. cleaved. When tested by moderately fresh sperm, cleavage rose to 45 per cent.

In experiment 6, the increased cleavage with fresh sperm was 57 to 73 per cent.

In experiment 7, the small increase with fresh sperm, *i. e.*, only 18 per cent. and 13 per cent. respectively, was due to the

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poor physiologic condition of the sperm as determined by tests with fresh eggs.

In every experiment, freshly liberated sperm raised the total cleavage from 0 or nearly 0 to 30, 40, 50 and even 80 per cent. of the eggs examined. There can be little doubt but that this means that the apparent death of the eggs (when no cleavage occurred) was really due to the precocious and rapid deterioration of the sperm, with moderately little deterioration of the eggs.

Hence to determine the exact physiologic condition of the eggs at any stage in the ageing cycle, and to determine the real longevity of the eggs, it is necessary to fertilize the eggs with freshly liberated sperm at each testing, as F. R. Lillie, '14, had done for a very different purpose. For example, the eggs of 4 females of experiment 1, Table V., when ⅓ hour old, gave 95

TABLE V.

SHOWS REDUCTION IN CLEAVAGE WHEN AGEING EGGS ARE FERTILIZED BY FRESH SPERM. SHOWS TRUE LONGEVITY OF EGGS. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Hours.		No. of ♂.	Female No.						Average Cleavage, Per Cent.
		♀.	♂.		1.	2.	3.	4.	5.	6.	
1	7/4	⅓	⅓	1	95						95
		20	½	2	50	43	95	80			67
		23	1⅔	2	0	4	0	3			2
		24	1/10	4	0	13	0	0			3
		25	1	4	0	1	2	0			1
		46	1	5	0	2	0	0			½
		48	1/10	6	0	0	0	0			0
2	7/5	23	1/10	1	7	0	0				1
		25	1	2	45	0	2				—
		50	1/10	1	0	0	0				0
3	7/7	46	⅓	1	6	0	1	0			1¾
		46	¼	2	0	0	1	0			¼
		47	¾	3	0	2	1	1			1
4	7/8	½	½	1					90	81	85
		23	⅓	1	0	22	43	25			22
		51	1/10	2	0	0	0	0			0

per cent. average cleavage; when 20 hours old, tested by fresh sperm, 67 per cent.; when 23 hours old, 7 per cent.; when 24 hours old, 3 per cent.; when 25 hours old, 1 per cent.; when 46 hours old, ½ per cent.; when 48 hours old, 0 per cent. These figures give a much more exact measure of the rate of deterioration of the eggs than when synchronously aged eggs and sperm

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were used. There was very little deterioration or dying until the 20th hour, and then a most rapid rate of destruction until the 24th hour, and a final residual minimum extending until the 48th hour. The eggs of female 1 died at 23 hours female 4 at 24 hours, female 3 at 25 hours, female 2 at 48 hours.

Similar results were obtained in experiments 2, 3 and 4. Forty-eight hours appeared to be the maximum longevity of the eggs of *Toxopneustes* under the given experimental conditions. Table V. gives the rate of deterioration of the eggs, Table III. of the sperm.

Arbacia.

The experiments were repeated with *Arbacia* eggs, and the inquiry was pushed further. The data are brought together in Table VI. The facts may be summarized as follows:

1. Eggs which no longer cleaved when fertilized by sperm of the same age, did cleave when fertilized by freshly liberated sperm. In experiment 1, the increase was from 0 to 39 per cent.; in experiment 2, from 0 to 56 per cent.; in experiment 3, from 0 to 44 per cent., etc. This is exactly as in *Toxopneustes*.

2. With further aging there occurred a second cycle of progressively decreasing cleavage. For example in experiment 5 24-hour eggs fertilized by 1-hour sperm averaged for the 6 females 42 per cent. When the same germ cells were 31 and 8 hours respectively, they averaged only 10 per cent. A similar secondary deterioration occurred in experiment 1. The rapid deterioration was due in both groups to the more rapid destruction of the sperm than of the eggs, as in *Toxopneustes*.

3. The real deterioration of the *eggs* was determined as in *Toxopneustes* by fertilizing aging eggs with freshly liberated sperm at each testing. It was found that the eggs were very long lived. In experiment 7, 20-hour eggs showed little deterioration, 96 per cent. cleaved. Even when 41 hours, there was little deterioration, for 81 per cent. cleaved. When 48 hours 19 per cent., when 65 hours 9 per cent., when 72 hours 1 per cent. The other experiments, viz: 2, 3, 5, 6, 7, 8, 9, 10, 11, etc., gave similar results. They showed the same characteristics as *Toxopneustes* eggs, namely, *a long period of little deterioration, which in Arbacia is about 20 hour under the given experimental*

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TABLE VI.

SHOWS REDUCTION IN CLEAVAGE WHEN AGING EGGS ARE FERTILIZED BY FRESH SPERM. SHOWS LONGEVITY OF EGGS OF *Arbacia*.

No.	Date.	Age of Germ Cells in Hours.		Female Number.							Time, Minutes.	Average Cleavage, Per Cent.	Loss Per Cent. per Hr.
		♀.	♂.	1.	2.	3.	4.	5.	6.	7.			
1	8/12a	4	4								60	80	
		28½	28½	0	0	0	0				60	0	
		28½	4½	29	33	28	67				60	39	
		48	½	2	½	0	0				60	1	1.8
		1½	1½								60	96	
2	8/16	23	23	0	0	0	0	0	0	0	60	0	
		24	4	81	79	82	0	65	82	8	60	56	
		42	1	30	0	80	0	0	0	0	90	15	
		42	⅙	77	0	72	0	0	0	0	60	21	
		48	1/10	90	3	79	0	0	0	0	90	24	
		65	¼	48	0	0	0	0	0	0	60	7	
		70	1/12	21	0	0	0	0	0	0	60	3	1.3
		88	1/12	0	0	0	0	0	0	0	60	0	
3	8/17	1½	1½	96	70	97					120	87	
		18½	18½	71	20	93						61	
		23	23	0	0	0						0	
		24	4	28	50	65						44	
		42	1	30	0	17						15	
		42	1/10	66	0	70						45	
		48	1/10	52	3	74						43	
		65	¼	45	4	1						16	
		70	1/12	18	6	5						9	1.1
4	8/17	46	1/12	40	41	67					60	43	
		7	25	0	0	0						0	
5	8/13	4	5	98	97	99	99	98	93		120	97	
		24	1	87	3	57	89	19	0			42	
		31	8	64	0	0	0	0	0			10	3.2
		49	1¼	0	0	0	0	0	0			0	
		49	1¼	0	0	0	0	0	0			0	
6	7/29	20	1/12	73	85						120	79	
		43		34	35							34	
		51		7	5							6	2.3
7	8/1	20–22	1/12	96	96						120	96	
		41		75	87							81	
		48		14	24							19	
		65		3	15							9	
		72		2									1.8
8	8/3	41	1/12	11	22						120	16	
		63		8								8	
		87		0								0	
9	8/9	48	1/12	9	36						120	22	
		68		0								0	
10	8/10	45	1/12	2	7						120	4	
		49		1	7							4	
		51		0	0							0	
		69		0	0							0	
11	8/11	20	1/12	89								89	
		40		7								7	

* Much cleavage, exact count not made.

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Table VI.—*Continued.*

No.	Date	Age of Germ Cells in Hours.		Female Number.							Time, Minutes.	Average Cleavage, Per Cent.	Loss Per Cent. per Hr.
		♀4.	♂4.	1.	2.	3.	4.	5.	6.	7.			
12	8/18	6	28	49	99	95	99				90	85	
		1	45½	0	0	0					60		
				91	47	97					120	81	
		5	49½	0	17	85					60		
				93	31	77					120	67	
		1/10	68	39	90	52	48				60		
				100	100	83	40				120	80	
		5	73	66	74	75	45				60		
				90	69	87	100				120	86	
		10	77	*	*	*							
		1/10	95	29	59	72	78				275	59	
13	8/12a	48	48	14							60		
				14							120	14	
	b	18	48	80							60		
				98							120	98	
	c	1/12	48	60							60		
				70							120	70	
	d	48	18	11							60		
				13							120	13	
	e	18	18	89							60		
				92							120	92	
	f	1/12	18	58							60		
				70							120	70	

conditions. Then there occurred a period of rapid deterioration, i. e., until the 40th hour, and thirdly a long period of minimum cleavage ranging from 50th to the 70th hour. Arbacia differs only in the rate of deterioration, which is several times slower than in *Toxopneustes.*

4. The longevity of *Arbacia* eggs was correspondingly longer than *Toxopneustes.* In experiment 1, the eggs of 2 out of 4 females ceased to cleave when 48 hours old. In experiment 2, the eggs of 4 females ceased to cleave at 42 hours; the fifth female at 65 hours, and the 6th female at 88 hours. In experiment 3, cleavage occurred in all 3 females at least till the 70th hour, etc., etc. The longevity ranged from 24 hours to beyond 72 hours, or about twice that of *Toxopneustes* eggs, due largely to the difference in temperature (about 10° C.) of the sea water.

Effect of Age Upon Sperm.

Freshly Liberated Eggs Fertilized by Aging Sperm. Longevity of Sperm.

In the reciprocal experiments, after the synchronously aging germ cells, no longer cleaved, the aging sperm were tested against freshly liberated eggs.

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In experiment 3 (Table IV.), when the *Toxopneustes* germ cells were both 8 hours old, 29 per cent. of the eggs cleaved; when 12 hours old 0 per cent. cleaved. When the sperm were 10 hours old, they were tested by fresh eggs, 10 minutes old, and gave an average cleavage of 10 per cent. This might suggest that the rate of deterioration in synchronously aging sperm cells was conditioned by the rate of deterioration of the sperm.

In experiment 5, 10–hour–old eggs fertilized by 10-hour-old sperm averaged 1 per cent. cleavage. When the sperm was tested by fresh eggs, the cleavage rose to 25 per cent.

Similarly in experiment 6, 11-hour old germ cells averaged but 5 per cent. cleavage; the old sperm by fresh eggs gave 91 per cent. cleavage. On the other hand, in experiment 7, 11-hour-old germ cells gave 0 per cent. cleavage, in both crosses.

The explanation for these apparently contradictory data is found in a triple comparison, namely,

1. Crossing of synchronously aged germ cells.
2. Crossing of aged eggs by fresh sperm.
3. Crossing of fresh eggs by aging sperm.

If this be done it becomes apparent at once that the results are due in largest part to the condition of the egg, subject only to the ability of the sperm no matter how deteriorated to penetrate the cortical layer and initiate development.

In experiment 5, Table IV., 10-hour-old germ cells gave 1 per cent. average cleavage. When the eggs 10½ hours old were fertilized by moderately fresh sperm, 45 per cent. cleaved; the reciprocal cross, viz,, fresh eggs by the old sperm, caused only 25 per cent. to cleave. When these fresh eggs were tested by fresh sperm, 28 per cent. cleaved, *i. e., approximately the same cleavage as when fertilized by 10-hour-old sperm.* Hence low cleavage in experiment *a* was due to the sperm, while in *c* and *d* it was due to the eggs.

In experiment 6, similar results were obtained. Eleven-hour-old germ cells gave 5 per cent. cleavage. Fresh eggs used to test the old sperm gave *91* per cent. cleavage. But when these fresh eggs were tested by fresh sperm, *approximately the same percentage cleaved as when fertilized by stale sperm.*

Experiment 7 is interesting because the fresh eggs used were in

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physiologically poor condition, and when fertilized by 11-hour-old sperm gave 0 per cent. cleavage. These same eggs tested by freshly liberated sperm gave the same results, namely 0 per cent.

Hence the results obtained by fertilizing fresh eggs by old sperm is determined in largest part by the physiologic condition of the eggs. The sperm plays but a minor rôle. One must be cautious, however, in any given experiment, in interpretng the results. In experiment 3, for example, the 10-hour-old sperm gave only 10 per cent. cleavage with fresh eggs. This low cleavage might have been due to the physiologically poor condition of the sperm or of the eggs. Actual tests showed that the fresh eggs were in poor condition and not the old sperm.

If the sperm deterioration is not reflected, or only to a very slight degree reflected, in a reduction in cleavage how does it happen that in all experiments *when aged eggs were fertilized by aged sperm the cleavage was far less than in either of the other two crosses.*

This result is probably due to a summation of effects, that due to the deteriorated condition of the eggs, and that due to the probable greater difficulty of aged sperm to initiate mitosis and cleavage. In the final analysis both are reducible to the condition of the egg.

Arbacia.

Further light was thrown upon these questions by the experiments with *Arbacia*.

Experiment 12, Table VI., is illustrative of the effect of aging upon the sperm. In this experiment after the germ cells no longer cleaved, the old sperm was tested against fresh eggs of 4 different females, over a long range of intervals. When the sperm were 28 hours old, the eggs of the 4 females averaged 85 per cent. cleavage (90 minutes after fertilization). When the sperm were 45½ hours old 81 per cent. of the fresh eggs of 4 other females cleaved; *when 73 hours old 86 per cent.; when 77 hours old "very many" (exact count not taken); when 95 hours old 59 per cent. of the eggs cleaved.* At this last observation the different females gave 29, 59, 72 and 78 per cent. cleavage. How much longer the sperm could have fertilized freshly liberated eggs was not determined. How much of the reduction (when the sperm

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were 95 hours old) was due to a physiologic deterioration of the sperm, comparable with that in the eggs, and how much the reduction was due to an excessive mortality, as a result of which there were insufficient numbers of effective sperm, was not determined. Both factors probably play important rôles, though I am inclined to believe that a differential mortality plays a major rôle.

In the above experiment and in others, *the decrease in cleavage with these very old sperm was amazingly small, far less than in eggs of like age.* It should be noted however that the sperm in these experiments were allowed to age in the "dry" or concentrated condition, without the addition of sea water, and in this condition their metabolism is at a minimum, hence the maximum longevity.

In experiment 13, various types of crosses corroborate the above conclusions. Eggs and sperm were fertilized at varying intervals until both germ cells were 48 hours old, at which time only 14 per cent. cleaved. This 48-hour-old-sperm was tested with 18-hour-old eggs and with $\frac{1}{12}$-hour-old eggs. The 18-hour eggs gave 98 per cent. cleavage; the $\frac{1}{12}$-hour eggs 70 per cent. Samples of the same eggs were also fertilized by sperm 18 hours old. The 48-hour eggs gave 13 per cent. cleavage; the 18-hour eggs 92 per cent. and the $\frac{1}{12}$-hour eggs 70 per cent. It will be at once evident that the sperm at both ages gave a *remarkably similar cleavage per cent. with fresh, with moderately old and with old eggs, i. e.*, the sperm have undergone little if any physiologic deterioration between 18 and 48 hours after liberation. The 48-hour eggs gave 14 per cent. cleavage by 48-hour sperm and 13 per cent. with 18-hour-old sperm. The 18-hour-old eggs gave 98 per cent. cleavage with 48-hour-old sperm and 92 per cent. with 18-hour sperm. The $\frac{1}{12}$-hour-old eggs gave 70 per cent. cleavage with 48-hour-old sperm and the same per cent. with 18-hour-old sperm.

The cleavage was determined essentially or exclusively by the condition of the eggs.

The decrease in cleavage in the fresh eggs was due as in *Toxopneustes* experiment to the poorer physiologic condition of the eggs at the time of liberation. *Good fresh sperm cannot cause physiologically poor eggs to cleave to any greater extent than old*

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sperm. Cleavage in physiologically good eggs is not decreased by old sperm.

From these data it will also be observed that the longevity of dry sperm was greater than the eggs in the given experimental conditions. In neither *Toxopneustes* nor in *Arbacia* was the maximum longevity of the sperm determined.

Effect of Age Upon Rate of Cleavage.

I have shown that the total number of eggs that cleaved in a given time after fertilization, decreased as the eggs aged. I propose now to show that there was, as F. R. Lillie, '14, indicated, a corresponding retardation of the rate of early cleavage, with increasing age of the egg; and in a later paper I propose to describe the irregular and abnormal character of cleavage in very aged eggs.

The rate of cleavage was ascertained by the total cleavage at three intervals, namely, (1) 40 minutes after fertilization, when the eggs first divided, (2) 1 hour after fertilization, when most and sometimes all the eggs had divided once, and (3) 2 hours after fertilization when all or very nearly all the eggs had divided at least once.

The data are brought together in Table VII.

In experiment 1, the eggs of 6 females were fertilized by one male, when the germ cells were 130, 240, 350 and 470 minutes old. A record was made at each interval, of the number of eggs that cleaved in 40, 60 and 120 minutes after each fertilization. In female no. 1, 54 per cent. cleaved within 40 minutes, when the germ cells were 130 minutes old; 37 per cent. cleaved in the same interval when 240 minutes old; 3 per cent. when 350 minutes old, 5 per cent. when 470 minutes old. *There was an undoubted decrease in the rate of first cleavage with age. The cleavage at the same ages 60 minutes after fertilization also showed a similar retardation with age of the germ cells*, for 78, 85, 51 and 59 per cent. respectively, cleaved. The total cleavage, namely 2 hours after fertilization, as I have shown before, also decreased with age. The corresponding figures were 82, 93, 92 and 77 per cent.

The average for all 6 females brings out forcibly the retardation and decrease in cleavage. The average for the germ cells 40

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TABLE VII.

SHOWS CHANGE IN RATE OF CLEAVAGE WITH AGE OF GERM CELLS. THE FIGURES REPRESENT THE PER CENT. CLEAVAGE IN INDICATED TIME. *Toxopneustes.*

No.	Date.	Age of Germ Cells in Minutes.	Female 1.			2.			3.			4.			5.			6.			Average Per Cent.			Ratio of Cleav in 40 Min.: 120 Min.	Ratio of Cleav in 60 Min.: 120 Min.
			40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min	40 Min.	60 Min.	120 Min.	40 Min.	60 Min.	120 Min	40 Min	60 Min.	120 Min.	40 Min.	60 Min.	120 Min.		
1	7/19	130	54	78	82	70	93	96	73	88	83	82	88	96	86	94	98	91	80	94	76	86	91	83	94
		240	37	85	93	83	99	99	46	56	84	67	76	89	78	78	93	82	67	91	65	76	91	71	83
		350	3	51	92	72	94	98	22	59	82	67	57	64	70	75	89	35	26	12	44	60	71	62	84
		470	5	59	77	17	46	64	16	43	70	0	0	4	64	75	82	1	6	80	27	39	51	53	76
2	7/12	17		98			98			87															
		42	56	77	80	34	78	76	18	30	31										36	62	62	58	100
		99	0	35	70	6	50	72	1	12	20										2	32	54	3	59
		161	4	15	33	6	18	38	1	11	14										3	14	28	10	50
		214	3	24	40	6	28	30	1	11	13										3	21	31	9	67
		294	3	14	18	½	17	14	0	0	2										1	10	11	9	90
		360	0	0	0	0	0	0	0	0	0										0	0	0	0	0
		420	0	0	0	0	0	0	0	0	0										0	0	0	0	0
3	7/14	20	½	93	92	0	32	61	17	98	98										5	74	83	6	89
		80	0	87	99	0	95	95	0	35	70										0	72	88	0	81
		140	2	91	98	2	95	95	2	99	97										1	95	96	1	98
		210	8	83	96	8	63	93	12	97	97										9	81	95	9	85
		280	6	92	92	32	77	82	5	86	87										14	85	87	16	97
		370	6	61	62	4	46	69	3	47	85										4	51	65	6	78
		440	0	25	62	0	20	59	0	51	87										0	32	69	0	46
		500	0	12	43	0	½	10	0	5	36										0	6	29	0	20
4	7/16	160	0	83[1]	50	0	74	74	0	26	81	0	28	96	0	20	18				0	38	53	0	
		300	0	39	34	0	60	61	0	54	37	0	34	92	0	38	34				0	45	49	0	
		500	3		28	0		8	5		20	4		29	1		66				2		26	7	

[1] Probably an error in recording.

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minutes after fertilization was *76, 65, 44 and 27 per cent.* for the 4 different ages of the germ cells. The average cleavage 60 minutes after fertilization, for the same ages, was *86, 76, 60 and 39 per cent.* and the total cleavage, *i. e.*, 2 hours after fertilization, was 91, 91, 71 and 51 per cent. respectively.

These observations suggest an explanation for the decreasing total cleavage with aging germ cells. For the decreased total may conceivably be due to a retardation of the division process, or an inhibition of this process, or both.

Records taken three hours after fertilization showed that the 2-hour observation really was the total cleavage; that very little and usually no additional cleavage occurred. *Hence the decreased total cleavage with aging germ cells was not an apparent decrease due to retardation, but a real decrease by inhibition of cleavage.*

We are concerned then with two symptoms or independent evidences of changes in the eggs caused by their aging. On the one hand, an increasing number of eggs were prevented from developing and, on the other hand, those that do develop, do so with increasing difficulty (retardation).

The relation between retardation and reduction in cleavage may be expressed as follows: When germ cells were 130 minutes old, 83 per cent. of the total number of dividing eggs cleaved within 40 minutes after fertilization; when the germ cells were 240 minutes old a less number, namely 71 per cent. of the cleaving eggs, divided in the same time. When the germ cells were 350 and 470 minutes old, 62 and 53 per cent. of the segmented eggs cleaved in 40 minutes.

In experiment 2, germ cells in poor condition were tested at 8 intervals, between ¼ hour and 7 hours, and the rate of cleavage noted at each interval. The results are essentially in accord with those of experiment 1. The per cent. cleaving in 40 minutes after each fertilization was 36, 2, 3, 3, 1, 0, 0 per cent.; for 60 minutes after fertilization, the record was 62, 32, 14, 21, 10, 0, 0; and the total cleavage 2 hours after fertilization was 62, 54, 28, 31, 11, 0, 0 respectively.

Experiment 4 is also quite in accord with the previous ones.

Experiment 3 differs from the above only in the increasing total cleavage for a brief period, followed by decreasing cleavage

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with further age. The rate of cleavage follows a similar cycle; *the rate of cleavage and the total cleavage increased with the age of the germ cells until they were 280 minutes old, after which the rate was retarded and the total cleavage declined.*

Arbacia.

With *Arbacia* eggs the rate of cleavage was ascertained by noting the per cent. of the eggs that reached the 4-cell stage in 60 minutes. The data are shown in Table VIII.

TABLE VIII.

SHOWS CHANGE IN RATE OF CLEAVAGE FROM THE PER CENT. OF EGGS THAT REACH THE 4-CELL STAGE IN 60 MINUTES. *Arbacia.*

No.	Date.	Age of Germ Cells in Hours.		Female 1.	2.	3.	4.	5.	6.	7.	Time, Minutes.
		♀.	♂.								
1	8/12	4	4	24	0	0	0	3			69
		23	23	1	0	0	0	1			67
		28	4½	2	6	5	12	36			67
		24	1	1	0	0	18	1			
		30½	8	0	0	0	0	0			
2	8/16	1½	1½	80	81	90	30	80	65	93	60
		18½	18½	3	0	0	0	0	0	1	
		24	4	3	10	1	0	6	1	1	
		42	$\frac{1}{2}$	1	0	0	0	0	0	0	
		48	$\frac{1}{8}$	3	0	2	0	0	0	0	
		65	$\frac{3}{4}$	4	0	0	0	0	0	0	
		70	$\frac{1}{10}$	2	0	0	0	0	0	0	
		88	$\frac{1}{10}$	0	0	0	0	0	0	0	
3	8/14b	1½	1½	42	22	67	77	90			60
		5	5	35	3	88	26	40			
4	8/14a	4	5	0	1	0	19	36			60
5	8/18	1	23	0	1	0	0				87
		5	49	0	8	0					60
		$\frac{1}{10}$	68	0	6	0					60
		5	73	5	5	3					60

In *Arbacia* as in *Toxopneustes* the total cleavage decreased and the early division process was increasingly retarded with increasing age of the germ cells. In experiment 3, for example, the eggs of 5 females were tested when their germ cells were 1½ hours old, and 59 per cent. of the eggs reached the 4-cell stage in 1 hour. When the germ cells were 5 hours old only 38 per cent. reached the 4-cell stage in the same time. In experiment 1 and 2, older eggs were used. The results were even more striking and in entire accord with the preceding experiments.

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Retardation is another symptom of the deterioration not of the sperm but of the eggs.

Correlation of Changes.

I have shown firstly that the freshly liberated eggs of freshly collected females of the same species varied considerably with respect to size, jelly layer, membrane formation and cleavage; secondly the range of variability for each of these types of changes was ascertained; thirdly, these changes were correlated; fourthly, by *these correlated phenomena one could accurately and conveniently ascertain the exact physiologic condition of the eggs of any female, at any time.*

Freshly liberated eggs from freshly collected females could be readily classified into physiologically "good," "poor" and "bad" eggs, on the basis of the correlated phenomena, as follows:

	Physiologically Good Eggs.	Physiologically Poor Eggs.	Physiologically Bad Eggs.
Size:	Norm for species.	Larger than norm.	Still larger or smaller than norm.
	Small deviation from norm.	Increasing deviation.	Still further deviation.
Jelly layer:	Maximum number with jelly layers 100–90 per cent.	Increasing loss of layer 96–70 per cent.	Further loss 70–40 per cent.
Membrane formation:	Formed within 2 minutes.	Increasingly retarded. 3–7 min.	None.
Cleavage:	Maximum number cleave 100–90 per cent.	Decreasing number 90–60 per cent.	Further decrease. 60–0.

Perhaps one example chosen at random may make the matter more definite. The freshly liberated eggs of *Arbacia* (Experiment 8–12) were practically normal in size, over 90 per cent. contained the jelly layer, fertilization membranes were formed in 1½ to 2 minutes, the cleavage was 95, 97, 96 and 94 per cent. in the eggs of 4 out of 5 females. The eggs of the fifth female were larger than the norm, less than 90 per cent. possessed jelly layers, no membranes were formed upon fertilization and only 18 per cent. cleaved. The eggs of the same females were tested by a second male, and gave the same size and jelly count, as before, and membranes appeared in 2, 2, 2½, 2½ minutes respectively. The cleavage count was 72, 69, 62 and 55 per cent. This retardation

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in membrane formation and reduction in cleavage was clearly due to the poor physiologic condition of the sperm. The eggs of the fifth female were likewise fertilized by this male and showed the same size and jelly count as before, but no membrane appeared and only 4 per cent. cleaved. By these tests it was definitely determined that the eggs of the first four females were in good physiologic condition, those of the fifth female in poor condition.

By these tests it is possible to state accurately whether any sample of eggs are "good," "poor" or "bad," and to state exactly to what degree of physiologic deterioration such sample of eggs may have reached. The words "good," "poor" or "bad" now have a specific meaning, in terms of definite measurable changes in size, loss of jelly, rate of membrane formation and cleavage, which changes symptomize and measure definite physiologic and morphologic changes in the eggs.

The freshly liberated eggs whose physiologic condition was determined, subsequently changed or aged or overripened, with respect to the same categories, namely, size, jelly layer, membrane and cleavage.

I have shown in this paper exactly the extent to which each of these categories varied with the aging of the germ cells. I wish now to emphasize the fact that the changes are correlated.

These correlated changes, each of which measures the degree of physiologic deterioration with age, may be briefly summarized in the following table:

	Freshly Liberated or Good Eggs.	Moderately Stale or Aged Eggs.	Very Stale or Aged Eggs.
Size:	Little deviation from the norm.	Increasing deviation in a plus direction.	Further deviation in plus or minus direction.
Jelly layer:	Maximum or close to maximum.	Decreasing.	Further decrease.
Fertilization membrane:	Within 2 minutes. Wide membranes.	Increasingly retarded. Increasingly narrow.	None.
Cleavage:	Maximum rate. Maximum number.	Increasing retardation. Decreasing numbers.	Further retardation. Further decrease.

Whatever the physiologic condition of the eggs when liberated, they undergo with age an increase in size, a decrease in the number

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possessing the jelly layer, a decrease in rate of membrane formation, and a decrease in cleavage, varying in degree with the condition of the eggs at the time of liberation. And one may predict the extent of the other changes from any known one or two. If size or membrane rate are known, one may predict very approximately the other symptoms of the physiologic condition of the eggs, such as jelly and cleavage, etc.

The discussion of these results will be postponed until further data will be presented, concerning other types of changes in very aged eggs. For the present I wish to draw attention to the following considerations.

1. The aging process or processes which are symptomized in the various changes described in this paper begin not with the liberation of the eggs, but upon their maturation, within the body of the mother. This was first suggested by Loeb, and I am in entire accord with his view. Hence it follows that chronologic age (time since liberation) affords but a poor idea of the real physiologic condition of the eggs either at liberation or at any interval thereafter.

2. Aging is a continuous process, beginning within the body and continuing (with somewhat accelerated rate) outside of the body, culminating ultimately in the death of the eggs.

3. Aging was manifested in a number of ways, any one of which may serve as an index of the physiologic condition, and the degree of deterioration. *The ensemble of the various indices forms a clear and unmistakeable measure of their condition and their deterioration.*

4. While no attempt has thus far been made to describe the nature of the chemico-physical processes involved in the aging of the eggs, they may nevertheless be accurately measured. It is now possible to measure very accurately the physiologic condition of the eggs at liberation, and at any interval of time thereafter, to measure accurately the rate of deterioration or senescence under given experimental conditions. It is also possible to measure the real longevity of the eggs or of the sperm.

5. These data, and those described in Part III., afford a basis for an understanding of the nature of the aging process, and of the means of controlling senescence of the germ cells.

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The discussion of the results and a more complete bibliography are given in Part III.

Summary.

The variation in size of the eggs, the per cent. with jelly layers, the rate of membrane formation and the total cleavage were ascertained for large numbers of freshly liberated eggs from freshly collected females, examined at different periods of the breeding season.

Three species of sea urchins were studied in this way, namely, *Toxopneustes* and *Hipponoë* of tropical waters, and *Arbacia* of the North Atlantic.

Large variations from the norm were observed and measured in all four categories. These variations were interpreted as indices of the physiologic condition of the eggs of each female at the time of liberation.

With the physiologic condition of freshly liberated eggs of a given female known, experiments were then instituted to ascertain the nature and extent of the changes in the germ cells, as they aged, or became overripe, under given optimum laboratory conditions.

The following is a brief statement of the changes in such aging germ cells, viz., changes in size, jelly layer, membrane formation and cleavage.

Change in Size.

Freshly liberated eggs in good physiologic condition varied but slightly from the norm. For details see text.

As these eggs aged, their volume increased continuously, until they cytolized or fragmented, and became smaller than the norm.

Freshly liberated eggs in poor physiologic condition either enlarged but little with age, or were directly reduced in size by cytolysis or fragmentation.

The nature, extent, and rate of change in size, depends upon the physiologic condition of the eggs when freshly liberated. Whatever the physiologic condition of the eggs may be, their senescence or physiologic deterioration can be very accurately measured by the degree of enlargement or reduction in their size.

Essentially the same results were obtained in the other two species.

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Change in Jelly Layer.

Practically all freshly liberated eggs in good physiologic condition possess a jelly layer. Those in poor condition have a correspondingly less per cent.

With age the jelly layer was lost in an increasing number of the eggs.

The rate of loss depended upon the condition of the eggs at the time of liberation (all other conditions remaining constant).

For fresh eggs in good physiologic condition, the rate of loss per hour was 0.81 and for equally fresh eggs in poor condition 2.65 or over 3 times as rapid.

Essentially similar results were obtained in all three species.

The loss of jelly layer was a second symptom and index of the extent and the rate of ageing or senescence of the eggs.

Change in Membrane.

Freshly liberated eggs in good physiologic condition formed fertilization membranes within two minutes. The rate depended partly upon the sperm but primarily upon the physiologic condition of the eggs.

With increasing age the time required to form the fertilization membranes was at first accelerated and later retarded. In very aged eggs no membranes were formed.

Freshly liberated eggs in poor physiologic condition showed direct retardation in the rate of membrane formation.

Aging eggs which no longer formed membranes when fertilized by old sperm could be made to form membranes with fresh sperm.

The rate of membrane formation is practically independent of the sperm. It is essentially determined by the condition of the egg.

As the eggs aged the membrane appeared closer and closer to the surface of the egg; it became thinner and ultimately none was formed.

These observations are essentially the same for all three species.

The rate and character of membrane formation affords a third means of measuring senescence in eggs.

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Change in Cleavage.

When freshly liberated eggs of *Hipponoë* and *Toxopneustes* in good physiologic condition were fertilized under the given optimum conditions at successive ages the total cleavage increased for a time and subsequently decreased,[1] and in extreme ageing none of the eggs segmented.

Cleavage then is an additional index of the degree of senescence or physiologic deterioration of the eggs.

There were two series of experiments, one in which the eggs and sperm aged synchronously, and the other in which they aged asynchronously. In both series the rate of decrease in cleavage with age was several times greater in *Toxopneustes* and *Hipponoë* than in *Arbacia*. This difference in rate of senescence as in rate of membrane formation, loss of jelly and change in size is due largely to differences in temperature, as well as to differences in HO concentration of the sea water, and to protoplasmic differences of the eggs.

When both germ cells aged synchronously the apparent longevity was about 11 hours for *Toxopneustes* and about 28 hours in *Arbacia*.

In asynchronous matings a more definite idea was obtained of the changes in the egg alone and in the sperm alone. When freshly liberated sperm were used to fertilize the eggs (of a female) at varying ages, with the precautions indicated in the text, the eggs showed progressively decreasing per cent. of cleavage (in *Arbacia*), but the rate of decrease was very much slower than when both germ cells aged synchronously. The decrease may be divided into three periods, the first a period of small decrease (about the first 20 hours in *Arbacia*) the second, a period of rapid and large decrease (between the 20th and 40th hour), and the third, a period of small decrease (between the 40th and 80th hour in *Arbacia*).

Eggs in poor physiologic condition at the time of liberation deteriorated at a correspondingly greater rate than physiologically good eggs.

[1] Freshly liberated eggs, of freshly collected *Arbacia*, in good physiologic condition, gave a maximum or nearly maximum cleavage and with ageing there was a direct decreasing total.

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The real longevity of the eggs depended upon the physiologic condition at time of liberation, the better, the longer lived, and vice versa.

The rate of cleavage was accelerated for a time and then retarded with age, more so in physiologically poor eggs, less in good eggs.

When ageing sperm fertilized freshly liberated eggs the decrease in cleavage was surprisingly small. Very little decrease occurred when sperm were 73 hours, only a little more when sperm were 95 hours old.

The explanation for the greater longevity of the sperm is found in the fact that the sperm aged in the "dry" condition, in which they are inactive and hence minimum metabolism.

The greater decrease in cleavage in synchronously ageing germ cells is due to a summation of the injurious effects upon the sperm and upon the eggs.

In very late stages in ageing of sperm, the reduction may be due not so much to physiologic deterioration as to insufficient numbers of sperm. There is ground for belief that no matter how old or deteriorated the sperm if they are active (alive) they can penetrate the egg. It is not so clear whether such aged sperm cause parthenogenesis or sexual development.

The change in size, jelly, membrane and cleavage with aging of germ cells, are accurate, convenient and corroborative indices of chemico-physical and morphologic changes in the egg as they age, and afford convenient measures of the loss in vitality, or physical deterioration. And one change may serve for this purpose. Their ensemble is convincing.

In the next study will be considered the changes in much older germ cells leading towards their cytolysis and death.

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